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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 21/04, C07K 14/00, C12N 15/12, G01N 33/53

A1

(11) International Publication Number: WO 98/56806

(43) International Publication Date: 17 December 1998 (17.12.98)

(21) International Application Number: PCT/US98/12263

(22) International Filing Date: 12 June 1998 (12.06.98)

(30) Priority Data:
60/049,452 12 June 1997 (12.06.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/049,452 (CIP)
Filed on 12 June 1997 (12.06.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A TRANSCRIPTION FACTOR COACTIVATOR PROTEIN, p/CIP

(57) Abstract

The present invention provides a substantially purified nucleic acid molecule encoding a p/CIP polypeptide, which regulates the activity of CBP/p300–dependent transcription factors. The invention also provides a substantially purified p–CIP polypeptide and active fragments thereof. In addition, the invention provides methods of identifying an effective agent that alters the association of a p/CIP polypeptide with a second protein. Further provided herein are methods of selectively inhibiting signal transduction pathways using an active fragment of a p/CIP polypeptide or a nucleic acid molecule encoding such an acive fragment.

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A TRANSCRIPTION FACTOR COACTIVATOR PROTEIN, p/CIP

This application claims the benefit of priority of U.S. provisional application 60/049,452, filed June 12, 1997, the entire contents of which are herein incorporated by reference.

ACKNOWLEDGMENT

This invention was made with government support under grant number DK39949-14A1 awarded by the National Institutes of Health and grant number CA52599-07 awarded by the National Cancer Institute. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to molecular biology and biochemistry and more specifically to a coactivator protein, p/CIP, which is involved in regulating gene expression by CBP/p300-dependent transcription factors, and to methods of using the coactivator protein to selectively regulate gene expression.

BACKGROUND INFORMATION

Regulation of gene expression is mediated by sequence-specific transcription factors that bind to target genes and activate or repress transcription. Many of these factors are controlled by extracellular signals that switch the factors between inactive and active states. Such signals can result in post-translational modification as observed, for example, with the members of the STAT family of transcription factors, or can result in ligand-induced conformational changes as

observed, for example, with members of the nuclear receptor family of transcription factors.

Coactivator proteins have been identified that are recruited to the active forms of such transcription factors and are required for their transcriptional effects. The coactivators, CBP and p300, for example, serve essential roles in transcriptional activation by several classes of regulated transcription factors, including nuclear receptors, STAT factors, AP-1 proteins, NF-κB and CREB. In addition, a more recently discovered family of proteins, termed nuclear receptor coactivator (NCoA) proteins, can interact with various nuclear receptors in a ligand-dependent manner and also can interact with CBP and p300.

Two members of the NCoA family of proteins, NCoA-1 and NCoA-2, appear to have relatively selective roles in mediating the transcriptional effects of nuclear receptors. Evidence indicates, however, that additional factors are required for the transcriptional activities of many CBP-dependent transcription factors, including STAT 1, AP-1 and CREB, and that complexes containing such coactivators, for example, CBP/p300 and NCoA, are involved in transmitting an activation signal to the promoter. Since CBP and p300-containing complexes appear to be limiting in cells, antagonistic interactions between signaling pathways can be due, at least in part, to competition for these complexes. Thus, a need exists to identify different classes of transcription factors that are regulated by a CBP-containing complex and to identify the coactivator proteins involved in such complexes. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a substantially purified nucleic acid molecule having a nucleotide sequence encoding a transcriptional coactivator protein, designated p/CIP, which binds to CBP/p300-dependent transcription factors and regulates their activity. For example, the invention provides a substantially purified nucleic acid molecule having the nucleotide sequence shown in Figure 1, which encodes murine p/CIP, and a nucleotide sequence complementary to that shown in Figure 1.

The invention also provides a substantially purified nucleic acid molecule encoding an active fragment of a p/CIP polypeptide, which has a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a p/CIP polypeptide. Such a nucleic acid molecule can encode, for example, an active fragment including a CBP interaction domain, such as a fragment having about amino acids 758 to 1115 of p/CIP shown in Figure 1, or a nuclear receptor interaction domain, such as a fragment having about amino acids 591 to 803 or about amino acids 680 to 740 of p/CIP shown in Figure 1.

Further provided herein is a substantially purified nucleic acid molecule having a nucleotide sequence encoding a full length mouse NCoA-2 protein, which is related to p/CIP. The invention also provides a substantially purified NCoA-2 active fragment, having a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a NCoA-2 polypeptide. Such a NCoA-2 active fragment can include, for example, a nuclear receptor interaction domain.

The invention also provides vectors comprising a nucleic acid molecule of the invention and host cells containing such vectors. In addition, the invention provides a substantially purified p/CIP nucleotide sequence having at least about 14 consecutive nucleotides of the nucleotide sequence shown in Figure 1, or a nucleotide sequence complementary thereto.

The present invention also provides a substantially purified p/CIP polypeptide, which can bind to a CBP/p300-dependent transcription factor and regulate its activity. For example, the invention provides a substantially purified p/CIP polypeptide having substantially the same amino acid sequence as p/CIP shown Figure 1. The invention additionally provides a substantially purified p/CIP active fragment having substantially the same amino acid sequence as a portion of a p/CIP polypeptide. A particularly useful p/CIP active fragment can include, for example, a CBP interaction domain or a nuclear receptor interaction domain, or can be an portion of a p/CIP polypeptide useful for eliciting production of an antibody that specifically binds to p/CIP.

The invention further provides a substantially purified NCoA-2 polypeptide having substantially the same amino acid sequence as amino acid sequence shown in Figure 2a. Active fragments of a NCoA-2 polypeptide of the invention also are provided herein.

The invention also provides anti-p/CIP antibodies that specifically bind to p/CIP, as well as p/CIP-binding fragments of such antibodies. The invention further provides anti-NCoA-2 antibodies and antigen binding fragments thereof. In addition, the invention provides cell lines producing anti-p/CIP antibodies or anti-NCoA-2 antibodies.

The present invention further provides methods of identifying an effective agent that alters the association of p/CIP or NCoA-2 polypeptide with a second protein, such as a nuclear receptor or a CBP, which associates with the p/CIP or NCoA-2 polypeptide *in vitro* or *in vivo*. The method includes the steps of contacting a p/CIP or NCoA-2 polypeptide with an agent under conditions that allow the p/CIP or NCoA-2 polypeptide to associate with the second protein, and detecting an altered association of the p/CIP or NCoA-2 polypeptide with the second protein. An agent that alters the association of p/CIP, for example, with a second protein can be a peptide, a polypeptide, a peptidomimetic or an organic molecule, such an effective agent being useful, for example, for modulating the level of transcription in a cell. For example, a peptide portion of p/CIP comprising a helical leucine-rich, charged domain (LCD), can inhibit the transcriptional activity of one type of nuclear receptor, such as the retinoic acid receptor, but not of a second, related nuclear receptor such as the estrogen receptor, whereas a second LCD of p/CIP can inhibit signal transduction induced by interferon γ, but not signal transduction induced by retinoic acid. Thus, selected peptide portions of p/CIP or of NCoA-2 can be valuable for regulating gene expression in a cell, and these and other effective agents can have therapeutic efficacy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence and deduced amino acid sequence of p/CIP.

Figures 2a to 2d provide a characterization of p/CIP and a related member of the NCoA family, NCoA-2.

Figure 2a compares the amino acid sequences of the full length murine p/CIP and murine NCoA-2 polypeptides. The conserved bHLH, PAS "A" domain, the nuclear receptor interaction domains and the minimal nuclear receptor and CBP interaction domains are boxed, and repeat motifs involved in critical interactions are bracketed.

Figures 2b and 2c provide western blot analyses of total cell extracts for p/CIP, NCoA-1 and NCoA-2 in various tissues and cell lines, showing widespread expression of all three proteins, although relative levels differ.

Figure 2d provides schematic diagrams showing regions of homology of p/CIP with NCoA-1 and NCoA-2. The asterisks refer to the repeated peptide motifs that appear to be of functional importance (see Figures 6 and 7).

Figure 3 shows the results of biochemical analysis of p/CIP and NCoA Factors.

Figure 3a demonstrates interactions between recombinant GST proteins and NCoA proteins from HeLa whole cell extracts detected using an anti-p/CIP antibody (left) or an anti-NCoA-1 antibody (right).

Figure 3b (left) shows co-immunoprecipitation of CBP/p300 and p/CIP. Anti-p/CIP, anti-NCoA-1 or anti-NCoA-2 IgG was incubated with HeLa whole cell extracts and immunocomplexes were separated by SDS-PAGE and probed using anti-CBP/p300 IgG. Figure 3b (right) shows the detection of CBP/p300 in supernatant following immunodepletion of whole cell extracts with specific anti-NCoA antibodies.

Figure 3c shows the results of yeast two-hybrid assays mapping regions of interaction between p/CIP and the CBP C-terminus (aa 2058-2170) or liganded estrogen receptor (LBD).

Figure 3d demonstrates that a common nuclear receptor interaction domain is found in p/CIP, NCoA-1 and NCoA-2 by yeast two-hybrid assay. Ligands (+) were estradiol (10^{-6} M), Triac (10^{-6} M) or retinoic acid (10^{-6} M).

Figure 3e shows p/CIP, NCoA-1 or NCoA-2 interactions with nuclear receptors *in vitro*. Recombinant GST-nuclear receptor proteins were incubated with whole cell extract in the presence(+) or absence (-) of ligand, then western blot analysis was performed using p/CIP-, NCoA-1- or NCoA-2-specific IgG.

Figure 3f shows the results of transcription activation studies, in which reporter genes containing the minimal prolactin promoter (P-36 luciferase), alone, or two copies of the indicated response elements, and plasmids expressing p/CIP, NCoA-1 or NCoA-2 were transfected into HeLa cells in the presence of the corresponding ligand. The effect of varying amounts of plasmid expressing GAL4 (1-147), GAL4-NCoA-1 or GAL4-p/CIP fusion proteins on a minimal $(UAS)_6$-dependent reporter are shown in the right panel.

Figure 4 demonstrates a role of P/CIP in the function of CBP-dependent transcription factors.

Figure 4a shows the effect of microinjection of affinity-purified anti-p/CIP IgG on ligand-dependent gene activation by RAR in Rat-1 cells.

Figure 4b shows experiments as in Figure 4a, but performed using minimal promoters with four copies of the estrogen (ERE), thyroid hormone (TRE) or progesterone (PRE) receptor response elements.

Figure 4c demonstrates that both CBP and p/CIP expression vectors are required to rescue anti-p/CIP IgG inhibition of RAR-dependent gene activation.

Figure 4d shows the effect of expression of the p/CIP core CBP interaction domain (947-1084) on RAR dependent transcription (left) or on SP-1 or CMV dependent transcription (right).

Figure 4e shows the effect of anti-p/CIP IgG (α p/CIP) on an interferon γ dependent promoter (GAS/LacZ)[12] (left) and the effect of p/CIP (aa 947-1084) on interferon γ stimulated transcriptions and failure of CBP expression vector to rescue this inhibition (right).

Figure 4f shows the effect of anti-NCoA-1 IgG (α 1) on GAS and cAMP-dependent (2 x CRE) promoters. All were performed at least three separate times, with >200 cells injected; error bars are ± 2 x SEM.

Figure 5 demonstrates a role for NCoA-1 and NCoA-2 in nuclear receptor function.

Figure 5a demonstrates that microinjection of affinity-purified anti-NCoA-1, but not of anti-NCoA-2, IgG blocked ligand-dependent gene activation by RAR (left), but did not inhibit expression of either the 6 x SP-1 or CMV-driven promoters (right).

Figure 5b shows experiments as in Figure 5a, except using minimal promoters with two copies of the estrogen (ERE) or T3R (TRE) response elements with less

profound effects upon progesterone (PRE) mediated transcription.

Figure 5c demonstrates that anti-NCoA-1 IgG blocked retinoic acid-dependent activation of the RARE/LacZ reporter was not rescued by CMV expression vectors expressing p/CIP or CBP; however, expression was fully rescued by CMV-NCoA-1 and b CMV-NCoA-2.

Figure 5d shows photomicrographs of rhodamine-stained injected cells and the corresponding protein of XGal staining.

Figure 6 shows the leucine-rich charged domains (LCD's) in p/CIP/NCoA/CBP.

Figure 6a shows that a repeated leucine-rich domain is required for protein-protein interactions between p/CIP, CBP, NCoA proteins and nuclear receptors. The sequence of some of these domains are noted, with the core hexapeptide motifs indicated by brackets. Helical wheels of NCoA-1 LCD2 and CBP LCD6 are shown.

Figure 6b shows that mutation of amino acids 70-73 in CBP (QLSELL→QLAAAA) resulted in a complete loss of ligand-dependent interaction with T3R.

Figure 6c shows results of the yeast two-hybrid assay of interactions between the NCoA-1 nuclear receptor interaction domains (aa 635-760) with nuclear receptors (left). Mutations of the LCD2 motif (RLHRLL→RLAAAA) abolished ligand-dependent interaction, while peptides encompassing LCD2 (37 amino acids "aa") alone or LCD6 (59 amino acids) were sufficient for ligand-dependent interaction (center). 24-mer peptides encompassing LCD1, LCD2 or a control peptide were tested for ability to inhibit binding of ^{35}S-labeled NCoA interaction domain

fragment (aa 635-760) to liganded RAR with TTNPB (1 μM) (right)

Figures 6d and 6e demonstrate the functional effect of plasmids expressing mutations in LCD2 (HRLL→AAAA) and LCD3 (RYLL→AAAA) of NCoA-1 on rescue of inhibition by microinjected anti-NCoA-1 IgG (α-1) on retinoic dependent transcription (Figure 6d) and on estrogen dependent transcription (Figure 6e).

Figure 7 demonstrates that distinct helical motifs block transcriptional effects of specific signal transduction pathways.

Figure 7a shows that a 19-mer peptide, corresponding to NCoA-1 LCD4, but not a control peptide (CBP-622, control P), inhibits retinoic acid induced, but not interferon γ induced gene expression.

Figure 7b shows the effect of microinjection of the N-terminal 22 amino acids of CBP (CBP N'-P1), a synthetic N-terminal CBP peptide, on retinoic acid and interferon gene activation events. A synthetic peptide corresponding to the identical peptide lacking the eight amino terminal amino acids (CBP N'-P2) failed to inhibit interferon-dependent gene activation events.

Figure 7c shows, similar to Figure 7b, that interferon γ inhibition of retinoic acid-dependent activation of the RARE/LacZ reporter (right panel) was fully abolished by co-injection of the CBP N'-P1 peptide, which had no effect on retinoic acid dependent inhibition of the GAS/LacZ reporter by activated retinoic acid reporter.

Figure 7d provides a model of p/CIP/CBP (p300) function, indicating that several signal transduction

pathways mediated by specific transcription factors require a functional p/CIP, CBP/p300 complex, and potentially p/CAF, with each partner required, but not sufficient, to mediate transcriptional effects. Nuclear receptor-specific requirements for distinct protein-protein associations via specific LCD's is suggested.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a substantially purified nucleic acid molecule encoding a transcriptional coactivator protein, designated p/CIP (p300/CBP/co-integrator-associated Protein), which binds to CBP/p300-dependent transcription factors and regulates their activity. For example, the invention provides a substantially purified nucleic acid molecule having the nucleotide sequence shown in Figure 1, which encodes p/CIP, and a nucleotide sequence complementary to that shown in Figure 1. As disclosed herein, p/CIP is a member of the NCoA (Nuclear receptor Co-Activator) gene family and is involved in regulating the transcriptional activities of various CBP-dependent transcription factors, including STAT 1, AP-1 and CREB. In addition, the invention provides a substantially purified nucleic acid molecule encoding a full length murine NCoA-2 polypeptide having the amino acid sequence shown in Figure 2a.

As used herein, the term "substantially purified," when used in reference to a nucleic acid molecule of the invention, means that the nucleic acid molecule is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with a nucleic acid molecule in a cell. A substantially purified nucleic acid molecule of the invention can be obtained, for example, by chemical

synthesis of the nucleotide sequence shown in Figure 1 or by cloning the molecule using methods such as those disclosed in Example I.

As disclosed herein, CBP is present in a complex with p/CIP, which is required for transcriptional activity of nuclear receptors and other CBP/p300-dependent transcription factors, including STAT and AP-1. The related nuclear receptor coactivator protein, NCoA-1, also is specifically required for ligand-dependent gene activation by nuclear receptors. p/CIP, NCoA-1, and CBP contain related leucine-rich charged helical interaction motifs that are required for receptor-specific mechanisms of gene activation. The disclosure of these leucine-rich motifs permits selective inhibition of distinct signal transduction pathways.

CBP and p300 are functionally conserved proteins that have intrinsic acetylase activity and serve essential roles in activation by a large number of regulated transcription factors, including nuclear receptors, CREB, AP-1, bHLH and STAT proteins (see, for example, Chakravarti et al., Nature 383:99-103 (1996); Kwok et al., Nature 370:223-226 (1994); Arias et al., Nature 370: 226-229 (1994); Eckner et al., Genes and Devel. 10(19): 2478-2490 (1996), each of which is incorporated herein by reference).

In addition to CBP and p300, a series of factors that exhibit ligand-dependent and AF2-dependent binding to nuclear receptor C-termini have been identified biochemically (see Halachmi et al., Science 264:1455-1458 (1994); Cavailles et al., EMBO J. 14:3741-3751 (1995); Kurokawa et al., Nature 377:451-454 (1995)) and by expression cloning (see Lee et al., Nature 374:91-94 (1995); Le Douarin et al., EMBO J. 14:2020-2033 (1995); Voegel et al., EMBO J. 15(14):3667-3675 (1996);

Hong et al., Proc. Natl. Acad. Sci., *USA* 93:4948-4952 (1996)). Two homologous factors, termed SRC-1/NCoA-1 and TIF-2/GRIP-1, increase ligand-dependent transcription by several nuclear receptors in cotransfection assays and constitute a nuclear receptor coactivator (NCoA) gene family.

p/CIP is a NCoA/SRC family member that forms a complex with CBP in a cell. Surprisingly, both p/CIP and NCoA-1 are required for the function of nuclear receptors, while p/CIP, but not NCoA-1, is required for function of other CBP-dependent transcription factors. A series of helical leucine-rich, charged residue-rich domains (LCD's) within these factors serve as interaction motifs that are involved in assembly of a coactivator complex and that contribute to the specificity of nuclear receptor activation events.

Studies of CBP, NCoA-1 and p/CIP have led to the identification of a series of helical motifs that are required for NCoA/nuclear receptor interaction, NCoA/CBP interaction and CBP/STAT interaction. Based on the identification of these helical motifs, corresponding peptides have been developed that, when injected into cells, selectively block signaling by retinoic acid, estrogen or interferon γ. These results demonstrate that the targeting of specific interaction motifs present in coactivator complexes can result in highly selective effects on patterns of gene expression. These observations further indicate that the utilization of specific interaction motifs by nuclear receptors can be altered by different classes of ligands, resulting in selective activities that can be of therapeutic benefit. Thus, the invention provides methods of identifying agents that modulate the activity of specific classes of transcription factors.

As disclosed herein, the cloned p/CIP cDNA is a novel member of the NCoA gene family. Like NCoA-1 (SRC-1) and NCoA-2 (TIF2, GRIP2), p/CIP interacts with several nuclear receptors in a ligand-dependent manner. Analysis of p/CIP, NCoA-1 and NCoA-2 has led to identification of a series of helical interaction motifs that mediate interactions between NCoA proteins and nuclear receptors, and a separate series of helical motifs that mediate interactions between NCoA proteins and CBP (see, for example, Figure 2a). Mutations within these motifs reduce the ability of the coactivator to mediate transcriptional activation. Remarkably, microinjection of peptides cornerbanding to specific helical motifs exert selective inhibitory effects on transcription by different classes of transcription factors. Using this strategy, effective agents such as peptides have been identified that selectively block STAT 1 activity but not nuclear receptor-dependent transcription. In addition, effective agents have been identified that selectively block retinoic acid receptor activity but not STAT 1 activity. These agents have been used to demonstrate that inhibitory effects of interferon γ on retinoic acid transcription involve the CBP/p/CIP coactivator complex.

The invention also provides vectors comprising a nucleic acid molecule of the invention and host cells containing such vectors. In addition, the invention provides nucleotide sequences that bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful, for example, as probes, which can identify the presence of a nucleic acid molecule encoding p/CIP in a sample or as antisense molecules, which can inhibit the expression of a nucleic acid molecule encoding a p/CIP.

A substantially purified nucleic acid molecule of the invention is exemplified by the nucleotide

sequence shown in Figure 1, which encodes p/CIP protein, also shown in Figure 1. Due to the degeneracy of the genetic code and in view of the disclosed amino acid sequence of a p/CIP protein, additional nucleic acid molecules of the invention would be well known to those skilled in the art. Such nucleic acid molecules have a nucleotide sequence that is different from that shown in Figure 1 but, nevertheless, encode the amino acid sequence shown in Figure 1. Thus, the invention provides a substantially purified nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence of murine p/CIP as shown in Figure 1. Similarly, the invention provides a substantially purified nucleic acid molecule encoding a full length NCoA-2 polypeptide having the amino acid sequence shown in Figure 2a.

As used herein, reference to "a nucleic acid molecule encoding p/CIP" indicates 1) the polynucleotide sequence of one strand of a double stranded DNA molecule comprising the nucleotide sequence that codes, for example, for p/CIP and can be transcribed into an RNA that encodes the coactivator, or 2) an RNA molecule, which can be translated, for example, into p/CIP. It is recognized that a double stranded DNA molecule also comprises a second polynucleotide strand that is complementary to the coding strand and that the disclosure of a polynucleotide sequence comprising a coding sequence necessarily discloses the complementary polynucleotide sequence. Accordingly, the invention provides polynucleotide sequences, including, for example, polydeoxyribonucleotide or polyribonucleotide sequences that are complementary to the nucleotide sequence shown in Figure 1 or to a nucleic acid molecule encoding p/CIP having the amino acid sequence shown in Figure 1.

As used herein, the term "polynucleotide" is used in its broadest sense to mean two or more nucleotides or nucleotide analogs linked by a covalent bond. The term "oligonucleotide" also is used herein to mean two or more nucleotides or nucleotide analogs linked by a covalent bond, although those in the art will recognize that oligonucleotides generally are less than about fifty nucleotides in length and, therefore, are a subset within the broader meaning of the term "polynucleotide."

In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. However, a polynucleotide also can comprise nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Such nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs (Lin et al., Nucl. Acids Res. 22:5220-5234 (1994); Jellinek et al., Biochemistry 34:11363-11372 (1995); Pagratis et al., Nature Biotechnol. 15:68-73 (1997)). The covalent bond linking the nucleotides of a polynucleotide generally is a phosphodiester bond. However, the covalent bond also can be any of numerous other bonds, including a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides (see, for example, Tam et al., Nucl. Acids Res. 22:977-986 (1994); Ecker and Crooke, BioTechnology 13:351360 (1995)).

Where it is desired to synthesize a polynucleotide of the invention, the artisan will know

that the selection of particular nucleotides or nucleotide analogs and the covalent bond used to link the nucleotides will depend, in part, on the purpose for which the polynucleotide is prepared. For example, where a polynucleotide will be exposed to an environment containing substantial nuclease activity, the artisan will select nucleotide analogs or covalent bonds that are relatively resistant to the nucleases. A polynucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced with recombinant DNA methods using an appropriate polynucleotide as a template. In comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally will be chemically synthesized, although an enzyme such as T7 polymerase can incorporate certain types of nucleotide analogs and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template (Jellinek et al., *supra*, 1995).

The invention also provides nucleotide sequences that can bind to a nucleic acid molecule encoding p/CIP. Such nucleotide sequences are useful, for example, as probes, which can hybridize to a nucleic acid molecule encoding a p/CIP and allow the identification of the nucleic acid molecule in a sample. A nucleotide sequence of the invention is characterized, in part, in that it is at least nine nucleotides in length, such sequences being particularly useful as primers for the polymerase chain reaction (PCR), and can be, for example, at least fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or twenty-one nucleotides in length. If desired, a nucleotide sequence of the invention can have at least twenty-five, thirty, thirty-five, forty or fifty nucleotides of the nucleotide sequence shown in Figure 1. Such a nucleotide sequence of the invention is useful as a hybridization probe or as

a primer for PCR and can be used, for example, to identify homologous nucleic acid molecules encoding p/CIP proteins in other eukaryotes, particularly other mammals, including humans.

As disclosed herein, p/CIP is a member of the NCoA protein family and, therefore, shares conserved structural regions with other members of this family. Thus, a nucleic acid molecule encoding p/CIP shares regions of substantial homology with a nucleic acid molecule encoding an NCoA protein such as NCoA-2. However, a comparison of the nucleic acid molecules encoding p/CIP and NCoA-2, for example, also will reveal nucleotide sequences that are unique to p/CIP, such nucleotide sequences being encompassed within the invention.

A substantially purified nucleotide sequence of the invention can comprise a portion of a coding sequence of a nucleic acid molecule encoding p/CIP or of a sequence complementary thereto, depending on the purpose for which the nucleotide sequence is to be used. In addition, a mixture of a coding sequence and its complementary sequence can be prepared and, if desired, can be allowed to anneal to produce double stranded oligonucleotides. The invention also provides antisense nucleic acid molecules, which are complementary to a nucleic acid molecule encoding p/CIP and can bind to and inhibit the expression of the nucleic acid molecule.

A nucleic acid molecule of the invention, including an antisense molecule, can be introduced into a cell by methods of transfection, or can be contained in a plasmid or viral vector, which can be introduced into the cell, such that the nucleic acid molecule is stably or transiently expressed (see, for example, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring

Harbor Laboratory Press 1989); Ausubel et al., Current Protocols in Molecular Biology (Green Publ., NY 1994), each of which is incorporated herein by reference). Accordingly, the invention provides vectors comprising a nucleic acid molecule of the invention and host cells, which are appropriate for maintaining such vectors. Vectors, which can be cloning vectors or expression vectors, are well known in the art and commercially available. An expression vector comprising a nucleic acid molecule of the invention, which can encode a p/CIP or can be an antisense molecule, can be used to express the nucleic acid molecule in a cell.

In general, an expression vector contains the expression elements necessary to achieve, for example, transcription of the nucleic acid molecule, although such elements also can be inherent to the nucleic acid molecule cloned into the vector. In particular, an expression vector contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible expression of a cloned nucleic acid sequence, a poly-A recognition sequence, and a ribosome recognition site, and can contain other regulatory elements such as an enhancer, which can be tissue specific. The vector also contains elements required for replication in a procaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, Meth. Enzymol., Vol. 185, D.V. Goeddel, ed. (Academic Press, Inc., 1990); Jolly, Canc. Gene Ther. 1:51-64 (1994); Flotte, J. Bioenerg. Biomemb. 25:37-42 (1993); Kirshenbaum et al.,

J. Clin. Invest 92:381-387 (1993), which is incorporated herein by reference).

A nucleic acid molecule, including a vector, can be introduced into a cell by any of a variety of methods known in the art (Sambrook et al., *supra*, 1989, and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1994), which is incorporated herein by reference). Such methods include, for example, transfection, lipofection, microinjection, electroporation and infection with recombinant vectors or the use of liposomes.

Introduction of a nucleic acid molecule by infection with a viral vector is particularly advantageous in that it can efficiently introduce the nucleic acid molecule into a cell *ex vivo* or *in vivo*. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the nucleic acid molecule contained in the vector to specific cell types. Viral or non-viral vectors also can be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A nucleic acid molecule also can be introduced into a cell using methods that do not require the initial introduction of the nucleic acid molecule into a vector. For example, a nucleic acid molecule encoding a p/CIP can be introduced into a cell using a cationic liposome, which also can be modified with specific receptors or ligands as described above (Morishita et al., J. Clin. Invest., 91:2580-2585 (1993), which is incorporated herein by reference; see, also, Nabel et al., *supra*, 1993)). In addition, a nucleic acid molecule can be introduced into a cell using, for example, adenovirus-polylysine DNA complexes (see, for example,

Michael et al., J. Biol. Chem., 268:6866-6869 (1993), which is incorporated herein by reference). Other methods of introducing a nucleic acid molecule into a cell such that the encoded p/CIP or antisense nucleic acid molecule can be expressed are well known (see, for example, Goeddel, *supra*, 1990).

Selectable marker genes encoding, for example, a polypeptide conferring neomycin resistance (Neo^R) also are readily available and, when linked to a nucleic acid molecule of the invention or incorporated into a vector containing the nucleic acid molecule, allow for the selection of cells that have incorporated the nucleic acid molecule. Other selectable markers such as that conferring hygromycin, puromycin or ZEOCIN (Invitrogen, Carlsbad CA) resistance are known to those in the art of gene transfer as markers useful for identifying cells containing the nucleic acid molecule, including the selectable marker gene.

A "suicide" gene also can be incorporated into a vector so as to allow for selective inducible killing of a cell containing the gene. A gene such as the herpes simplex virus thymidine kinase gene (TK) can be used as a suicide gene to provide for inducible destruction of such cells. For example, where it is desired to terminate the expression of an introduced nucleic acid molecule encoding p/CIP or an antisense p/CIP nucleic acid molecule in cells containing the nucleic acid molecule, the cells can be exposed to a drug such as acyclovir or gancyclovir, which can be administered to an individual.

Numerous methods are available for transferring nucleic acid molecules into cultured cells, including the methods described above. In addition, a useful method can be similar to that employed in previous human gene transfer studies, where tumor infiltrating lymphocytes

(TILs) were modified by retroviral gene transduction and administered to cancer patients (Rosenberg et al., New Engl. J. Med. 323:570-578 (1990); see, also, Anderson et al., U.S. Patent No. 5,399,346, issued March 21, 1995, each of which is incorporated herein by reference).

The present invention also provides a substantially purified p/CIP polypeptide, which forms a complex with CBP/p300 in a cell and regulates CBP/p300-dependent transcriptional activity. A p/CIP polypeptide of the invention is exemplified herein by murine p/CIP, which is a protein of about 152 kDa that has a conserved amino-terminal basic helix-loop-helix domain, PAS "A" domain, a serine/ threonine-rich region and a carboxy-terminal glutamine-rich region. Murine p/CIP is related to SRC-1/NCoA-1 and NCoA-2/TIF-2, showing overall amino acid identity of 31% and 36%, respectively. As disclosed herein, p/CIP is required for transcriptional activation by CBP-dependent transcription factors, including nuclear receptors such as the retinoic acid receptor, estrogen receptor, thyroid receptor and progesterone receptor, and other CBP-dependent transcription factors such as STAT 1 (see Example I).

Thus, the present invention provides a substantially purified p/CIP polypeptide. Such a polypeptide can have, for example, substantially the same amino acid sequence as murine p/CIP shown in Figure 1 (see, also, Figure 2a). Further provided herein is a substantially purified p/CIP active fragment having substantially the same amino acid sequence as a portion of a p/CIP polypeptide. Such an active fragment can include, for example, a CBP interaction domain or a nuclear receptor interaction domain. LCD peptide portions of p/CIP also are provided (see Example I).

The term "substantially purified," as used herein in reference to a polypeptide or fragment thereof, means that the polypeptide or polypeptide fragment is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with a polypeptide in a cell.

As used herein, the term "p/CIP" or "p/CIP polypeptide" means the polypeptide referred to herein as "p300/CBP/CoIntegrator-associated Protein" and includes the murine p/CIP polypeptide shown in Figure 1. As described above, murine p/CIP displays homology to SRC-1/NCoA-1 and NCoA-2/TIF-2, sharing basic helix-loop-helix domains, a PAS "A" domain, serine/threonine-rich region and glutamine-rich region with NCoA-1 and NCoA-2.

The term p/CIP encompasses murine p/CIP and is intended to include related polypeptides having substantial amino acid sequence similarity to this polypeptide. Such related polypeptides will exhibit greater sequence similarity to p/CIP than to SRC-1/NCoA-1 or to NCoA-2/TIF-2 and include alternatively spliced forms of p/CIP and isotype variants of the amino acid sequence shown in Figure 1. The term p/CIP also encompasses homologous polypeptides obtained from different mammalian species, such as a human homolog of the murine p/CIP polypeptide disclosed in Figure 1. A p/CIP polypeptide generally has an amino acid identity of greater than about 40%, preferably greater than about 50%, more preferably greater than about 60%, and can have an amino acid identity of greater than about 70%, 75%, 80%, 85%, 90% or 95% with the murine p/CIP amino acid sequence disclosed in Figure 1.

As used herein, the term "substantially the same amino acid sequence," when used in reference to a

p/CIP amino acid sequence, is intended to mean the amino acid sequence shown in Figure 1, or a similar, non-identical sequence that is considered by those skilled in the art to be a functionally equivalent nucleotide or amino acid sequence. Thus, a polypeptide that has substantially the same amino acid sequence as a p/CIP polypeptide can have one or more modifications such as amino acid additions, deletions or substitutions relative to the amino acid sequence shown in Figure 1, provided that the p/CIP polypeptide retains at least one biological activity of a native p/CIP polypeptide.

Therefore, it is understood that modifications can be made without destroying the biological function of a p/CIP polypeptide. Also, for example, genetically engineered variants of p/CIP either alone or fused to heterologous proteins that retain at least one measurable activity in binding to a CBP protein, binding to a nuclear receptor, activity in retinoic acid, estrogen, thyroid or progesterone dependent transcription, activity in other CBP-dependent transcription, or other inherent biological activity fall within the definition of a p/CIP polypeptide.

It is understood that modifications of primary amino acid sequence can result in polypeptides which have substantially equivalent, enhanced or reduced function as compared to the murine p/CIP sequence set forth in Figure 1. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental such as through mutation in hosts harboring a p/CIP encoding nucleic acid molecule. All such modified polypeptides are included in the definition of a p/CIP polypeptide as long as at least one biological function of a p/CIP polypeptide is retained. Further, various molecules can be attached to a p/CIP polypeptide including, for example, other polypeptides, carbohydrates, lipids, or

chemical moieties using methods well known in the art. Such modifications are included within the term "p/CIP polypeptide," as defined herein.

Further provided herein is an isolated active fragment of a p/CIP polypeptide, which includes substantially the same amino acid sequence as a portion of a p/CIP polypeptide. As used herein, the term "active p/CIP fragment" means a peptide or polypeptide which has substantially the same amino acid sequence as a portion of a p/CIP polypeptide, provided that the fragment retains at least one biological activity of a p/CIP polypeptide. As defined herein, an active fragment generally has an amino acid sequence of about 15 to about 400 contiguous residues and can have, for example, an amino acid sequence of at least about 18, 20, 25, 30, 35, 40, 50, 100, 150, 200, 250, 300, 350 or 400 contiguous residues. A particularly useful active fragment has from about 80 to about 150 amino acids. A biological activity of a p/CIP polypeptide that is retained by an active p/CIP fragment can be, for example, measurable activity in binding to a CBP protein, binding to a nuclear receptor, activity in retinoic acid, estrogen, thyroid or progesterone dependent transcription, activity in other CBP-dependent transcription, or other inherent biological activity.

An isolated p/CIP active fragment of the invention can include, for example, a CBP interaction domain. Such a CBP-binding active fragment can have, for example, an amino acid sequence that is identical or substantially the same as a portion of p/CIP shown in Figure 1, for example, substantially the same as about amino acids 758 to 1115 of p/CIP, about amino acids 947 to 1084 of p/CIP, or about amino acids 163 to 610 of p/CIP shown in Figure 1. Additional p/CIP active fragments having a CBP interaction domain can readily be

identified, for example, using yeast two-hybrid assays or microinjection assays, as set forth in Example I. As disclosed herein, such an active fragment can block CBP-dependent gene activation, for example, interferon-γ or TPA stimulated gene activation or retinoic acid dependent gene activation.

A substantially purified p/CIP active fragment of p/CIP can include a nuclear receptor interaction domain. Such a nuclear receptor-binding active fragment of p/CIP can have, for example, an amino acid sequence that is identical or substantially the same as a portion of p/CIP shown in Figure 1, and can bind a nuclear receptor such as the estrogen receptor, for example, in a ligand-dependent manner. An example of a p/CIP active fragment having a nuclear receptor interaction domain is a fragment having substantially the same amino acid sequence as about amino acids 591 to 803 of p/CIP or about amino acids 680 to 740 of p/CIP shown in Figure 1.

Also provided herein is a novel member of the nuclear receptor co-activator family designated NCoA-2. As disclosed herein, murine NCoA-2 is a polypeptide of about 160 kDa that interacts with a 100 amino acid region in the carboxy termini of CBP (amino acids 2058-2170), as well as with the liganded estrogen receptor. Thus, the invention provides a substantially purified NCoA-2 polypeptide having substantially the same amino acid sequence as the amino acid sequence shown in Figure 2a. In addition, the invention provides a substantially purified NCoA-2 active fragment having substantially the same amino acid sequence as a portion of a NCoA-2 polypeptide. An active fragment of a NCoA-2 polypeptide can include, for example, a nuclear receptor interaction domain.

As used herein, the term "NCoA-2" or "NCoA-2 polypeptide" is intended to mean a polypeptide having substantial similarity to the murine NCoA-2 polypeptide shown in Figure 2a. Like a p/CIP polypeptide, a NCoA-2 polypeptide has a basic helix-loop-helix domain, a PAS "A" domain, a serine/threonine-rich region and a glutamine-rich region.

The term NCoA-2 encompasses murine NCoA-2 and is intended to include related polypeptides having substantial amino acid sequence similarity to this polypeptide. Such related polypeptides will exhibit greater sequence similarity to NCoA-2 than to SRC-1/NCoA-1 or to p/CIP and include alternatively spliced forms of NCoA-2 and isotype variants of the amino acid sequences shown in Figure 2a. The term NCoA-2 also encompasses homologous polypeptides obtained from different mammalian species, although the human TIF-2 and GRIP-1 polypeptides described in Voegel et al., EMBO J. 15:3667-3675 (1996) and Hong et al., Proc. Natl. Acad. Sci. *USA* 93:4948-4952 (1996), each of which is incorporated by reference herein, are explicitly excluded from the term NCoA-2 polypeptide as defined herein. A NCoA-2 polypeptide generally has an amino acid sequence having an amino acid identity of greater than about 70%, preferably greater than about 75%, more preferably greater than about 80%, and can have an amino acid identity of greater than about 85%, 90% or 95% with the murine NCoA-2 amino acid sequence disclosed in Figure 2a.

An active fragment of a p/CIP or NCoA-2 polypeptide can be produced by any of several methods well known in the art. For example, an active fragment of the invention can be produced by enzymatic cleavage of a p/CIP polypeptide using a proteolytic enzyme such as trypsin, chymotrypsin or the like, or a combination of such enzymes. The resulting enzymatic digestion

subsequently can be purified using well known methods. An active fragment also can be produced using methods of solution or solid phase peptide synthesis or can be expressed from a nucleic acid molecule such as a portion of the coding region of the nucleic acid sequence shown in Figure 1, or can be purchased from a commercial source.

The invention also provides an LCD peptide portion of p/CIP, which includes a helical leucine-rich, charged domain (LCD) and which can inhibit the transcriptional activity of one type of nuclear receptor, such as the retinoic acid receptor, but not of a second, related nuclear receptor such as the estrogen receptor. An LCD peptide portion of p/CIP also can selectively inhibit signal transduction induced by interferon γ without inhibiting signal transduction induced by retinoic acid. Thus, an LCD peptide portion of p/CIP or of another NCoA can be useful for regulating gene expression in a cell.

An LCD peptide portion of p/CIP or NCoA-2 is characterized, in part, as containing one or more copies of the consensus core sequence, LXXLL, where L is leucine and X is independently selected to be any amino acid. Preferably, an LCD peptide portion contains at least three copies of the consensus core sequence LXXLL. An LCD peptide portion of p/CIP can include, for example, one or more of the following amino acid sequences: KGHKKLLQLLTCS, LLQEKHRILHKLLQN, KKNNALLRYLLDRDD, LRNSLDDLLGPPS or RALLDQLHTFL. An LCD peptide portion of NCoA-2 can include, for example, one or more of the following amino acid sequences: KGQTKLLQLLTTK, SLKEKHKILHRLLQD, KKENALLRYLLDKDD, FGSSPDDLLCPHP or GALLDQLYLAL. An LCD peptide portion of p/CIP or of NCoA-2 can be a helical domain with amphipathic characteristics and can have a length of eight, nine,

ten, twelve, fourteen, sixteen, twenty, forty, sixty, eighty or more residues.

As used herein, the term "amino acid" includes both amino acids and amino acid equivalents. An amino acid equivalent is a compound which departs from the structure of a naturally occurring amino acid, but which has substantially the structure of an amino acid, such that it can be substituted within a peptide or protein which retains its biological activity. Thus, for example, amino acid equivalents can include amino acids having side chain modifications or substitutions, and also can include related organic acids, amides or the like. Amino acid equivalents include amino acid mimetics, which are those structures which exhibit substantially the same spatial arrangement of functional groups as amino acids but do not necessarily have both the α-amino and α-carboxyl groups characteristic of amino acids.

The invention also provides anti-p/CIP antibodies and anti-murine NCoA-2 antibodies, as well as antigen binding fragments of such antibodies. In addition, the invention provides cells lines such as isolated cell lines that produce antibodies of the invention, particularly monoclonal antibodies. As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. With regard to an anti-p/CIP antibody, for example, the term "antigen" means a p/CIP protein, polypeptide or peptide portion thereof. An anti-p/CIP antibody, or antigen binding fragment of such an antibody, is characterized by having specific binding activity for p/CIP or a peptide portion thereof of at least about 1×10^5 M^{-1}. An anti-p/CIP antibody can have specific binding activity for p/CIP without binding other NCoA polypeptides such as

NCoA-1 or NCoA-2. Fab, $F(ab')_2$, Fd and Fv fragments of an anti-p/CIP antibody, which retain specific binding activity for p/CIP, are included within the definition of an antibody. Similar antibodies can be identified with respect to the full length murine NCoA-2 polypeptide disclosed herein.

In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995); each of which is incorporated herein by reference).

Anti-p/CIP antibodies can be raised using as an immunogen a substantially purified full length p/CIP protein, which can be prepared from natural sources or produced recombinantly, or a peptide portion of a p/CIP polypeptide as defined herein, including synthetic peptides as described above. A non-immunogenic peptide

portion of p/CIP can be made immunogenic by coupling the hapten to a carrier molecule such bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane, *supra*, 1988.

Particularly useful antibodies of the invention are those that bind to uncomplexed p/CIP, but not to a p/CIP in a complex, for example, with CBP, and, conversely, those that bind to the complexed form of p/CIP, but not to the uncomplexed form. An anti-p/CIP antibody is useful, for example, for determining the presence or level of a p/CIP in a tissue sample, which can be a lysate or a histological section. The identification of the presence or level of a p/CIP in the sample can be made using well known immunoassay and immunohistochemical methods (Harlow and Lane, *supra*, 1988). An anti-p/CIP antibody also can be used to substantially purify p/CIP from a sample and, in addition, can be used to copurify a protein such as a transcription factor that is complexed with the p/CIP polypeptide. An anti-p/CIP antibody can be used to detect a p/CIP polypeptide in a sample of cells or in an organism.

A kit incorporating an anti-p/CIP antibody, which can be specific for the complexed or uncomplexed form of p/CIP, can be particularly useful. Such a kit can contain, in addition to an anti-p/CIP antibody, a reaction cocktail that provides the proper conditions for performing the assay, control samples that contain known amounts of p/CIP and, if desired, a second antibody specific for the anti-p/CIP antibody.

A protein such as an anti-p/CIP antibody, as well as p/CIP or a peptide portion thereof, can be labeled so as to be detectable using methods well known in the art (Hermanson, "Bioconjugate Techniques" (Academic Press 1996), which is incorporated herein by reference; Harlow and Lane, *supra*, 1988; chap. 9). For example, a protein can be labeled with various detectable moieties including a radiolabel, an enzyme, biotin, or a fluorochrome or fluorescent moiety, such as a green fluorescent protein (see U.S. Patent No. 5,625,048; WO96/23810; WO97/28261; PCT/US97/12410; and PCT/US97/14593, each of which is incorporated herein by reference). Reagents for labeling a protein such as an anti-p/CIP antibody can be included in a kit containing the protein or can be purchased separately from a commercial source.

Following contact, for example, of a labeled antibody with a sample such as a tissue homogenate or a histological section of a tissue, specifically bound labeled antibody can be identified by detecting the particular moiety. Alternatively, a labeled second antibody can be used to identify specific binding of an unlabeled anti-p/CIP antibody. A second antibody generally will be specific for the particular class of the first antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labeled using a detectable moiety as described above. When a sample is labeled using a second antibody, the sample is first contacted with a first antibody, which is an anti-p/CIP antibody, then the sample is contacted with the labeled second antibody, which specifically binds to the anti-p/CIP antibody and results in a labeled sample.

Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are

well known in the art. In addition, monoclonal antibodies can be obtained using methods that are well known and routine to the skilled person (Harlow and Lane, *supra*, 1988). Essentially, spleen cells from a p/CIP-immunized mouse can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labeled p/CIP protein to identify clones that secrete anti-p/CIP monoclonal antibodies. Hybridomas expressing anti-p/CIP monoclonal antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies, which are useful, for example, for preparing standardized kits as described above. Similarly, a recombinant phage that expresses, for example, a single chain anti-p/CIP antibody also provides a monoclonal antibody that can used for preparing standardized kits.

A monoclonal anti-p/CIP antibody can be used to prepare anti-idiotypic antibodies, which presents an epitope that mimics the epitope recognized by the monoclonal antibody used to prepare the anti-idiotypic antibodies. Where the epitope recognized by the monoclonal antibody includes, for example, an LCD, the anti-idiotypic antibody can act as an inhibitor of p/CIP binding to CBP or p/CIP binding to a transcription factor, thus providing a means to regulate a signal transduction pathway.

The present invention also provides a method of identifying an effective agent that alters the association of a p/CIP polypeptide with a second protein such as CBP or a transcription factor, or that alters the formation of a complex containing two or three of these proteins. The method includes the steps of contacting a p/CIP polypeptide and a second protein with an agent under conditions that allow the p/CIP polypeptide to

associate with the second protein and detecting an altered association of the p/CIP polypeptide and said second protein. The altered association indicates that the agent is an effective agent. In a method of the invention, the p/CIP polypeptide can have, for example, the amino acid sequence shown in Figure 1, and the second protein can be, for example, a CBP protein, a nuclear receptor or a CBP/p300-dependent transcription factor. An altered association can be detected, for example, by measuring the transcriptional activity of a reporter gene. In a method of the invention, a p/CIP polypeptide can be contacted with an agent *in vitro* or in a cell, including a prokaryotic cell such as a yeast cell and a eukaryotic cell, such as a mammalian cell, for example, a human cell.

The present invention further provides a method of identifying an effective agent that alters the association of a NCoA-2 polypeptide with a second protein. The method includes the steps of contacting a NCoA-2 polypeptide and a second protein with an agent under conditions that allow the NCoA-2 polypeptide to associate with the second protein and detecting an altered association of the NCoA-2 polypeptide and said second protein.

As used herein, the term "second protein" refers to a protein that specifically associates with a p/CIP or NCoA-2 polypeptide. It is recognized, however, that p/CIP and NCoA-2 can associate with more than one additional protein at the same time to form a complex. Thus, a second protein is exemplified herein by CBP proteins, by nuclear receptors and by other CBP/p300-dependent transcription factors, which form a complex with p/CIP or NCoA-2. Effective agents that alter the association, for example, of p/CIP, CBP and a transcription factor can be extremely valuable in that

the agent can modulate transcriptional activity of the transcription factor.

The term "agent," as used herein, means a biological or chemical compound such as a simple or complex organic molecule, a peptide, a peptidomimetic, a polypeptide, a nucleic acid, a chemical or a small molecule. The screening assays described herein are particularly useful in that they can be automated, facilitating high through-put screening of randomly or rationally designed agents or libraries of agents, such as chemicals, small molecules, drugs, peptides, peptidomimetics or polypeptides, in order to identify those agents that alter the association of p/CIP or NCoA-2 with a second protein. If desired, an agent can be screened individually, or can be screened in combination with other agents, for example, in a library.

As used herein, the term "associate" or "association," when used in reference to a p/CIP or NCoA-2 polypeptide and a second (or second and third) protein means that the p/CIP or NCoA-2 polypeptide and the second protein have a binding affinity for each other such that they form a bound complex *in vivo* or *in vitro*, including in a cell in culture or in a reaction comprising substantially purified reagents. For convenience, the term "bind" or "interact" is used interchangeably with the term "associate.

The screening assays disclosed herein provide a method of identifying an "effective agent," which is an agent that can increase or decreased the affinity of an association between a p/CIP or NCoA-2 polypeptide and a second protein and that has presumptive therapeutic activity. The term "modulate" or "alter," as used herein in reference to the association of a p/CIP or NCoA-2 polypeptide and one or two other proteins, means that the

affinity of the association is increased or decreased. Effective agents that can alter such association and, therefore, complex formation of p/CIP, CBP and a transcription factor, can be useful for modulating a signal transduction pathway and, therefore, expression of genes in the pathway. One skilled in the art understands that an effective agent that alters the association of p/CIP with a second protein such as a CBP protein may, additionally alter the association of other proteins with p/CIP. Alternatively, an effective agent can selectively alter the association of, for example, p/CIP with a CBP protein without altering the association of p/CIP with other proteins.

One skilled in the art understands that an effective agent can function directly or indirectly and by a variety of mechanisms to alter the association of a p/CIP polypeptide, or NCoA-2 polypeptide, with a second protein. An effective agent can function, for example, as a competitor of the binding interaction between a p/CIP polypeptide and a second protein, or between a NCoA-2 polypeptide and a second protein. For example, a peptide or peptidomimetic that mimics the structure of the CBP interaction domain or the nuclear receptor interaction domain of a p/CIP polypeptide can be an effective agent that decreases the affinity of the association of a p/CIP polypeptide with a second protein, as can be a fragment of a CBP protein or nuclear receptor that alters the association of p/CIP with a second protein. A peptide portion of p/CIP comprising an LCD, for example, amino acids 947 to 1084 of p/CIP (see Figure 2A) is an example of such an agent, since the peptide inhibits, for example, retinoic acid-dependent gene activation (see Example I). Additional peptide effective agents, which can be peptides as small as about five amino acids, can be identified, for example, by screening a peptide library (see, for example, Ladner et

al., U.S. Patent No: 5,223,409, which is incorporated herein by reference) using one of the assays described herein.

An effective agent also can bind to a p/CIP or NCoA-2 polypeptide at a site distant from the site of interaction, thereby altering the three-dimensional conformation of the polypeptide such that the affinity of the association with a second protein is increased or decreased. An effective agent also can produce an altered association by promoting a modification such as phosphorylation of a p/CIP or NCoA-2 polypeptide. In addition, an effective agent can sequester or alter the subcellular localization of a p/CIP or NCoA-2 polypeptide, thereby modulating the effective concentration of the polypeptide and the extent to which the polypeptide can associate with a second protein.

A variety of *in vivo* and *in vitro* screening assays for detecting an altered association are well known in the art including, for example, the two hybrid assay, coimmunoprecipitation assays, reporter assays and other well known methods such as equilibrium dialysis. One skilled in the art understands that methods for distinguishing the specific association of a p/CIP, for example, and a second protein from a non-specific interaction are routine and, generally, include performing the appropriate control experiments to demonstrate the absence of non-specific protein binding.

An effective agent can be identified by an altered level of reporter gene transcription as compared to a control level of transcription in the absence of the agent. A particularly useful reporter gene is cytosolic β-lactamase, which can be detected by the CCF2/AM substrate, as described in Tsien et al. (U.S. patent No. 5,741,657, which is incorporated herein by reference).

If desired, a reporter gene can encode a protein expressed, for example, on the cell surface, and an altered level of reporter gene transcription detected by FACS analysis.

A two-hybrid system, such as the yeast two hybrid system, can be particularly useful for screening a panel of agents in order to detect an altered association of a p/CIP or NCoA-2 polypeptide with a second protein (see Example I). Using a two hybrid system, an effective agent is identified by an altered level of transcription of a reporter gene (see Fields and Song, Nature 340:245-246 (1989), which is incorporated herein by reference). For example, the level of transcription of a reporter gene due to the bridging of a DNA-binding domain p/CIP or NCoA-2 polypeptide hybrid and a transactivation domain-second protein hybrid can be determined in the absence and presence of an agent.

In some cases, an agent may not be able to cross the yeast cell wall and, therefore, cannot enter a yeast cell to alter the association of a p/CIP or NCoA-2 polypeptide with the second protein. The use of yeast spheroplasts, which are yeast cells that lack a cell wall, can circumvent this problem (Ausubel et al., *supra*, 1994), which is incorporated herein by reference). In addition, an agent, upon entering a cell, may require "activation" by a cellular mechanism, which may not be present in yeast. Activation of an agent can include, for example, metabolic processing of the agent or a modification such as phosphorylation of the agent, which can be necessary to convert the agent into an effective agent. In this case, a mammalian cell line can be used to screen a panel of agents. A transcription assay such as the yeast two hybrid system described in Example I can be adapted for use in mammalian cells using well known methods (see, for example, Fearon et al., Proc. Natl.

Acad. Sci., USA 89:7958-7962 (1992), which is incorporated herein by reference; see, also, Sambrook et al., *supra*, 1989; Ausubel et al., *supra*, 1994).

An altered association also can be detected using an *in vitro* screening assay. *In vitro* screening assays can utilize, for example, p/CIP or a p/CIP fusion polypeptide such as a histidine-p/CIP fusion protein. For use in an *in vitro* screening assay, the p/CIP or p/CIP fusion polypeptide should have an affinity for a solid substrate as well as the ability to associate with a second protein. Convenient solid substrates include columns, beads, filters and other materials well known in the art. If desired, the solid substrate can contain a covalently attached anti-p/CIP antibody. Alternatively, when a fusion polypeptide such as a His-p/CIP fusion polypeptide is used in the assay, a nickel chelate substrate, which is bound by the histidine component of the fusion protein, can be used (Invitrogen, Carlsbad, CA). Other fusion polypeptide systems are well known in the art and commercially available, including glutathione-S-transferase (GST) fusion polypeptides, which can be immobilized on a glutathione affinity resin (Stratagene, La Jolla, CA) or using an anti-GST antibody (DAKO, Carpinteria, CA); "FLAG" fusion polypeptides, which can be immobilized on a substrate using anti-FLAG antibody; "AU" fusion polypeptides, which can be immobilized on a substrate using anti-AU antibody, commercially available from Berkeley Antibody Co., Richmond, CA; or Myc tag fusion polypeptides, which can be immobilized on a substrate using anti-Myc antibody, commercially available from Invitrogen. As an alternative to immobilization of the p/CIP or NCoA-2 polypeptide, the second protein can be immobilized on a solid substrate using a fusion protein strategy or antibody, as described above.

An *in vitro* screening assay can be performed by allowing a p/CIP or NCoA-2 polypeptide or p/CIP or NCoA-2 fusion polypeptide, for example, to bind to a solid substrate, then adding the second protein, and an agent to be tested. Control reactions, which do not contain an agent, can be performed in parallel. Incubation is performed under suitable conditions, which include, for example, an appropriate buffer concentration, pH, incubation time and temperature. Subsequently, the association of the p/CIP or NCoA-2 polypeptide and the second protein in the absence and presence of an agent can be detected, for example, by attaching a detectable moiety such as a radionuclide, fluorescent or antigenic label to the p/CIP or NCoA-2 polypeptide, and measuring the amount of label that is associated with the solid support. By comparing the amount of association in the presence of an agent as compared to the control level of association, an effective agent can be identified.

As set forth above, agents to be screened according to a method of the invention can include a variety of biological or chemical compounds such as organic molecules, peptides and peptidomimetics, polypeptides or nucleic acids. In particular, such agents to be screened include fragments of p/CIP or NCoA-2 polypeptides and fragments of CBP proteins or nuclear receptors. Such fragments can be produced by chemical or proteolytic cleavage of the isolated polypeptide. Methods for chemical and proteolytic cleavage and for purification of the resultant polypeptide fragments are well known in the art as described above. (See, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990), which is incorporated herein by reference).

A large collection, or library, of chemicals or small molecule drugs also are agents that can be screened according to a method of the invention. Polypeptide libraries, random polypeptides, or polypeptides of interest also are agents that can be screened for activity as disclosed herein. Polypeptide libraries include, for example, tagged chemical libraries comprising peptides and peptidomimetic molecules. Polypeptide libraries also comprise those generated by phage display technology. Phage display technology includes the expression of polypeptide molecules on the surface of phage as well as other methodologies by which a protein ligand is or can be associated with the nucleic acid which encodes it. Methods for production of phage display libraries, including vectors and methods of diversifying the population of peptides which are expressed, are well known in the art (see, for example, Smith and Scott, Methods Enzymol. 217:228-257 (1993); Scott and Smith, Science 249:386-390 (1990); and Huse, WO 91/07141 and WO 91/07149, each of which is incorporated herein by reference). These or other well known methods can be used to produce a phage display library which can be screened, for example, with one of the disclosed assays to identify an effective agent that alters the association of a p/CIP or NCoA-2 polypeptide with a second protein.

A peptide portion of p/CIP comprising a helical leucine-rich, charged domain (LCD), can inhibit the transcriptional activity of one type of nuclear receptor, such as the retinoic acid receptor, but not of a second, related nuclear receptor such as the estrogen receptor, whereas a second LCD of p/CIP can inhibit signal transduction induced by interferon γ, but not signal transduction induced by retinoic acid. Thus, selected peptide portion of p/CIP or of an NCoA can be useful for selecting regulating gene expression in a cell. Because

of the central nature of p/CIP, NCoA proteins, CBP/p300 and the various transcription factors that form the complexes disclosed herein, an agent such as an LCD peptide can be used to treat various pathologic conditions. For example, the transcriptional activity of CBP/p300-dependent proteins belonging to the AP-1 and ets families of transcription factors can be specifically inhibited, thereby providing a means to reduce or prevent the severity of inflammatory diseases or of cancer. In addition, selective activities of nuclear receptors can be potentiated or inhibited, providing beneficial effects in patients suffering from inflammatory disorders, breast cancer or osteoporosis. Also, inhibition of NF-κB dependent transcription can provide a benefit to patients having an inflammatory disorder or atherosclerosis. Thus, an agent that alters the association of a p/CIP or of NCoA-2 polypeptide with CBP/p300 or with a CBP/p300-dependent transcription factor, or alters the ability of such proteins to form a complex, is useful as a medicament for treating a pathologic condition.

The ability of nuclear receptor coactivator (NCoA) polypeptides such as p/CIP to interact with nuclear receptors in a ligand-dependent manner provides the basis of a method for identifying a ligand of a nuclear receptor from a library of one or more test agents. If desired, a nuclear receptor corepressor (NCoR) can be used in place of a NCoA polypeptide. A method of the invention can be useful for identifying a ligand for a nuclear receptor having a previously identified physiologic ligand or for identifying a ligand of an orphan receptor having no previously identified ligand. Thus, the invention provides a method for identifying a ligand for a nuclear receptor, including the steps of contacting a NCoA polypeptide, or nuclear receptor binding fragment thereof, and a nuclear receptor with an agent under conditions that allow the NCoA

polypeptide to associate with the nuclear receptor; and detecting an altered association between the NCoA polypeptide, or nuclear receptor binding fragment thereof, and the nuclear receptor, where an increased association indicates that the agent is an agonistic ligand of the nuclear receptor and a decreased association indicates that the agent is an antagonistic ligand of the nuclear receptor. One skilled in the art understands that the altered association in the presence of the agent is compared to the association in the absence of the agent.

The term "NCoA polypeptide," as used herein, means a nuclear receptor coactivator protein that is characterized, in part, as containing one or more "LXXLL" motifs and by its ability to mediate ligand-dependent nuclear receptor activation. A NCoA polypeptide can be, for example, a SRC-1/NCoA-1, p/CIP or NCoA-2 polypeptide, or a polypeptide having substantial similarity to one of these polypeptides. A NCoA polypeptide can have, for example, at least about 30% amino acid identity with a SRC-1/NCoA-1, p/CIP or NCoA-2 polypeptide and, further, can have at least about 40%, 50%, 60%, 70%, 80% or 90% amino acid identity with a SRC-1/NCoA-1, p/CIP or NCoA-2 polypeptide.

The agent to be tested for agonist or antagonist activity can be provided in purified form, or in impure form as a pool of different agents. As described above, an agent can be a biological or chemical compound such as a simple or complex organic molecule, a peptide, a peptidomimetic, a polypeptide or a nucleic acid. The nuclear receptor can be, for example, a steroid hormone receptor, retinoid receptor or fatty acid metabolite receptor. Retinoic acid receptors, estrogen receptors, progesterone receptor and thyroid receptors are examples of nuclear receptors useful in the claimed

methods. The NCoA polypeptide can be, for example, a p/CIP, NCoA-1 or NCoA-2 polypeptide, or an active fragment of a NCoA polypeptide with nuclear receptor binding activity. Active p/CIP and NCoA-2 polypeptides including a nuclear receptor interaction domain have been described hereinabove. Such a fragment can be, for example, a LCD peptide that contains one or more LXXLL motifs. In the presence of an agent that is an agonist, the nuclear receptor can undergo a conformational change, whereby there is an increased association of the nuclear receptor with the NCoA polypeptide or nuclear receptor interaction domain thereof, or with another coactivator containing one or more LXXLL motifs.

In one embodiment, the nuclear receptor can be immobilized on a solid substrate, for example, by expressing the nuclear receptor as a GST fusion protein and capturing the fusion protein on a glutathione affinity matrix. The nuclear receptor fusion protein can be incubated, for example, with labeled NCoA polypeptide such as labeled p/CIP polypeptide in the presence of an agent to be tested. Following incubation and subsequent washing of the glutathione affinity matrix, specifically bound NCoA polypeptide can be detected quantitatively, semi-quantitatively, or qualitatively. In the presence of a ligand that is an agonist, the association of the p/CIP or other NCoA polypeptide with the GST matrix is increased. Conversely, in the presence of a ligand that is an antagonist of the nuclear receptor, the association of the NCoA polypeptide with the GST matrix is decreased.

As set forth above, a NCoA polypeptide such as p/CIP can be labeled with a variety of labels including fluorescent labels or radiolabels such as ^{35}S-labeled amino acids, which can be incorporated by *in vitro* translation using a rabbit reticulocyte translation system. A LCD peptide containing at least one LXXLL

motif also can be used, if desired, in place of or in addition to, the NCoA polypeptide. Such a LCD peptide can be modified to contain N- or C-terminal tyrosine residues that do not substantially influence interaction with the nuclear receptor but which can be conveniently labeled, for example, using radioiodination. A short sequence tag suitable for phosphorylation of an LCD peptide with ^{32}P-ATP also can be used as a label. Fluorescent detection, for example using green fluorescent protein, can be particularly useful in the methods of the invention. Useful fluorescent detection methods include florescence polarization as well as fluorescence resonance energy transfer (FRET)-based assays. FRET-based assays are particularly advantageous for high throughput screening approaches since such assays are homogeneous and do not require a washing step and, in addition, can be useful for detecting nuclear receptor interactions within a cell.

In the methods of the invention, the ligand to be identified can be an antagonist. The present invention provides, for example, a method of identifying an antagonist of a nuclear receptor. The method includes the steps of contacting a NCoA polypeptide or nuclear receptor interaction domain thereof and a nuclear receptor with an agonist of the nuclear receptor and an agent under conditions that allow the NCoA polypeptide or nuclear receptor interaction domain thereof to associate with said nuclear receptor; and detecting an altered association of the NCoA polypeptide or nuclear receptor interaction domain thereof and the nuclear receptor, where a decreased association indicates that the agent is an antagonist of the nuclear receptor. An agent that is an antagonist can compete with the agonist for binding to the nuclear receptor without inducing the conformation change required for interaction of the NCoA polypeptide, or nuclear receptor interaction domain thereof, and the

nuclear receptor. As described above, a LCD peptide containing one or more LXXLL motifs can be substituted for a NCoA polypeptide in the methods of the invention.

In a further embodiment, the invention provides a method of identifying a ligand with mixed agonist and antagonist properties with respect to a particular nuclear receptor. Such a method includes the steps of: contacting a first NCoA polypeptide, or nuclear receptor interaction domain thereof, and a nuclear receptor with an agent to form a first complex; detecting an altered association of the first complex in the presence and absence of the agent; contacting a second NCoA polypeptide, or nuclear receptor interaction domain thereof, and a nuclear receptor with the agent to form a second complex; and detecting an altered association of the second complex in the presence and absence of the agent, wherein an increased association of the first complex combined with a decreased association of the second complex or a decreased association of the first complex combined with an increased association of the second complex indicates that the agent is a ligand of the nuclear receptor having mixed agonist and antagonist activity. A ligand identified by this method can be particularly useful since it can exhibit different effects on nuclear receptor function in different cell types and can be useful for differentially modulating different classes of transcription factors.

The following example is intended to illustrate but not limit the present invention.

EXAMPLE I

IDENTIFICATION AND CHARACTERIZATION OF p/CIP

This example provides methods for isolating and characterizing the nucleic acid molecule encoding p/CIP,

which regulates the activity of CBP/p300-dependent transcription factors. Additional details related to these methods are provided in Torchia et al., June 1997, at http://www.Nature.com, which is incorporated herein by reference.

A. MATERIALS AND METHODS

1. Isolation of Interacting Proteins

Expression cloning was performed, using a ^{32}P-labeled GST-CBP (2058-2170) or ^{32}P-labeled GST-ER ligand binding domain probe in the presence of 10^{-6} M estradiol (Kamei et al., Cell 85:1-12 (1996), which is incorporated herein by reference). cDNA's corresponding to p/CIP, NCoA-1 and NCoA-2 were assembled into PCMX and tested by *in vitro* translation, generating products which all migrated at approximately 160 kDa. Databank accession numbers for p/CIP and NCoA-2 sequences are AF000581 (p/CIP) and AF000582 (NCoA-2), each of which is incorporated herein by reference.

2. Yeast Two-hybrid Interaction Assays

The yeast strain EGY 48, the LexA-β-galactosidase reporter construct (PSH 18-34) and the B42 parental vectors (pEG 202 and pJG 4-5) were all previously described (Gyuris et al., Cell 75:791-803 (1993), which is incorporated herein by reference; Kamei et al., *supra*, 1996). Nuclear receptor ligand binding domains and various CBP fragments were subcloned into PEG 202 bait vector. DNA fragments encompassing the entire p/CIP-NCoA-1 or NCoA-2 proteins were generated either by using an appropriate restriction digest or by PCR and subcloned into pJG 4-5 prey vectors. EGY 48 cells were transformed with the *lac Z* reporter plasmid pSH 18-34

with the appropriate bait and prey vectors and plated out on -Ura-His-Trp medium containing 2% galactose. Isolated yeast colonies were allowed to grow in the same liquid medium, followed by assaying for β galactosidase, as previously described (Ausubel et al., *supra*, 1994).

3. Transient Transfections and Reporter Assays

Transfection experiments were conducted in either HELA or CV-1 cells using the standard calcium phosphate procedure. Typically, 1 ug of a RARE- or ERE-driven luciferase reporter were cotransfected with 1 ug of the indicated vectors. The final DNA concentration was adjusted to 10 ug/60 mm dish, incubated for 24 hr, then the appropriate ligands were administered for 24 hr at a concentration of 10^{-6} M. Alternatively, cotransfection experiments were conducted using a PCMX p/CIP, NCoA-1 or PCR-generated NCoA-1 fragments fused to the GAL 4 DNA binding domain (aa 1-147). Cells were transfected with 1 ug of a $(UAS)_6$-luciferase reporter and the indicated concentrations of GAL4 fusion proteins, then harvested 48 hrs later.

4. Affinity Purified NCoA Antibodies and Peptides

cDNA fragments corresponding to p/CIP (544-851), NCoA-1 (424-789) or NCoA-2 (787-1129) were subcloned into the pM vector containing an in-frame His tag and recombinant His-tagged proteins were generated and purified by nickel chelate chromatography. The purified recombinant proteins were injected into rabbits and antibodies were generated and affinity purified using standard procedures (Harlow and Lane, *supra*, 1988. Peptide sequences were generated (Research Genetics) and

confirmed by mass spectrosopy analysis, including NCoA-1 LCD1 (aa 631-647); NCoA-1 LCD2 (687-706); NCoA-1 LCD4 (aa 907-926); CBP N'-P1 (aa 1-19); and CBP N'-P2 (aa 8-19).

5. Interaction Assays, Immunoprecipitations and Enzymatic Assays

Whole cell extracts were prepared by lysing the cells in NET-N buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM DTT, 0.1% NP-40 and protease inhibitors (0.2 mM PMSF, 10 ug/ml each of leupeptin, pepstatin and aprotinin), centrifuged at 30,000 x g for 1 hr at 4°C and the supernatant was stored at -80°C until use.

GST-RAR (143-462), GST-ER(251-595) and GST-CBP(2058-2170) were generated as described (Kamei et al., *supra*, 1996). 25 ul of GST SEPHAROSE beads containing 10 ug of the GST recombinant proteins were incubated in the presence or absence of the appropriate ligand for 30 min at room temperature, followed by the addition of 1 mg of cell extract and incubated for an additional 1 hr at 4°C. The complexes were then centrifuged, washed three times in NET-N buffer, separated by SDS-PAGE and western blotted with the appropriate antibodies (1 ug/ml). For co-immunoprecipitation assays, 1 mg of cell extract was incubated in the presence of 2 ug of p/CIP or NCoA antibody for 2 hr at 4°C. The immune complexes were precipitated with protein A SEPHAROSE (50% w/v). Protein complexes were separated by SDS-PAGE (Laemmle, Nature 227:680-685 (1970), which is incorporated herein by reference) and western blotted using 1 ug/ml of an anti-CBP/P300 monoclonal antibody (UBI). For *in vitro* competition assays, the indicated peptides were incubated

with *in vitro* translated NCoA-1 prior to GST interaction with RAR.

6. Mutagenesis

Mutations in NCoA-1 and CBP were introduced by site-directed mutagenesis using the quick change mutagenesis kit according to the manufacturers instructions (Stratagene; La Jolla CA). Double stranded oligonucleotides were designed such that the wild type sequence corresponding to amino acids 695 to 698 and amino acids 756 to 759 in pCMX NCoA-1 and pJG4-5-4 NCoA-1(aa 635-760) were substituted with alanines. A similar protocol was used to replace amino acids 70 to 73 in PJG4-5 CBP(aa 1-101).

7. Single Cell Microinjection Assay

Insulin-responsive Rat-1 fibroblasts were seeded on acid washed glass coverslips at subconfluent density and grown in MNE/F12 medium supplemented with 10% fetal bovine serum, gentamicin and methotrexate. Prior to the injection, the cells were rendered quiescent by incubation in serum-free medium for 24-36 hr. Plasmids were injected into the nuclei of cells at a final concentration of 100 mg/ml. Peptides were injected at a concentration of 200 mM. Either preimmune IgG of the appropriate species or antibodies directed against p/CIP, NCoA-1 or NCoA-2 were co-injected and allowed the unambiguous identification of the injected cells.

Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately 1 hr after injection, the cells were stimulated, where indicated, with the appropriate ligand. In the case of rescue experiments, the cells were stimulated with ligand 6 hr

after injection, to allow protein expression. After overnight incubation, the cells were fixed, then stained to detect injected IgG and β-galactosidase expression (Rose et al., J. Cell. Biol. 119:1405-1411 (1992), which is incorporated herein by reference; Kamei et al., *supra*, 1996). Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG.

B. RESULTS

1. Identification of Novel Members of the Nuclear Receptor Co-Activator Family

The initial expression screening strategy for identifying members of the p160 gene family was based on the observation that the biochemically-identified p160 proteins interacted with a 100 amino acid region in the C-termini of CBP (aa 2058-2170), as well as the liganded estrogen receptor (ER; Ogryzko et al., Cell 87:953-960 (1996), which is incorporated herein by reference). This strategy allowed isolation of the previously reported NCoA-1/SRC-1 protein and of a second related factor, NCoA-2 (Figure 2a), which has a molecular mass of 159.6 kDa and appears to be the murine homologue of the human TIF-2, a portion of which has been recently reported as GRIP-1 (Voegel et al., EMBO J. 15(14):3667-3675 (1996); Hong et al., Proc. Natl. Acad. Sci. *USA* 93:4948-4952 (1996), each of which is incorporated herein by reference). In addition, a related factor was identified and is designated herein as p300/CBP/Co-Integrator-Associated Protein.

p/CIP is a 152 kDa protein that is highly related to SRC-1/NCoA-1 and NCoA-2/TIF-2, showing an overall amino acid identity of 31% and 36%, respectively (Figure 2a). p/CIP has a conserved N-terminal bHLH, PAS "A" domain (50-60% amino acid identity), a serine/

threonine rich region, and a C-terminal glutamine-rich region, each of which also is present in NCoA-1 and NCoA-2. Western blot analysis indicates that p/CIP, NCoA-1 and NCoA-2 are widely expressed in adult tissues and in all cell lines evaluated (Figures 2b and 2c).

2. A CBP/pCIP Complex

To evaluate the association of p/CIP, NCoA-1, and NCoA-2 with CBP and nuclear receptors, GST-CBP(2058-2170) was used to affinity purify interacting proteins from HeLa cell extracts. p/CIP was consistently observed by immunoblotting using affinity purified anti-p/CIP IgG, whereas much smaller amounts of NCoA-1 were detected following immunoblotting with anti NCoA-1 IgG (Figure 3a). Similarly, immunoprecipitations from whole cell extracts using excess antisera selective for each protein, followed by immunoblotting with anti-CBP/p300 antibody, demonstrated that the vast majority of CBP/p300 coprecipitated with p/CIP, although small amounts of NCoA-1- and NCoA-2-associated CBP were detected (Figure 3b). Conversely, the amount of CBP/p300 remaining in the supernatant fraction following immunodepletion with anti-NCoA-1 IgG remained unchanged, while a significant fraction of CBP was removed following immunodepletion with anti-p/CIP IgG (Figure 3b). These results indicate that p/CIP forms a complex with CBP in the cell.

To further define the CBP interaction domain in p/CIP, deletion mutants were generated and tested against CBP(2058-2170) using a yeast two-hybrid assay. The major CBP interaction domain was located between amino acids 758 to 1115 of p/CIP, with an internal 200 amino acid domain still capable of interacting. Interestingly, a less pronounced interaction was observed with the N-terminal region containing the PAS "A" domain

(Figure 3c). A single nuclear receptor interaction domain (aa 591-803) was localized N-terminal of the CBP/p300 interaction domain (Figure 3c). Further mapping delineated a minimal nuclear receptor interaction region encompassing amino acids 680-740 in p/CIP that was sufficient for binding to the liganded nuclear receptors. Comparable regions in NCoA-1 and NCoA-2 were found to mediate interactions with both CBP/p300 and nuclear receptors (Figure 3d). GST pull-down assays of whole cell extracts revealed that p/CIP, NCoA-1, and NCoA-2 interacted with GST-ER and GST-RAR in a ligand-dependent manner (Figure 3e).

Cotransfection with NCoA-1/SRC-1 or NCoA-2/TIF-2 expression vectors clearly potentiated ligand-dependent activation events (generally 3-fold to 8-fold), while cotransfection with p/CIP expression plasmids resulted in minimal or no activation effects (Figure 3f, left). In addition, when full length cDNA's were fused to GAL4(1-147), the activation observed by GAL-NCoA-1 was significantly stronger than GAL-p/CIP (Figure 3f, right). Cotransfection of CBP and NCoA-1 or NCoA-2 expression vectors resulted in variable synergy (data not shown), consistent with previous findings reported for SRC-1 (Smith et al., Proc. Natl. Acad. Sci., *USA* 93:8884-8888 (1996)).

To investigate the functional roles of p/CIP, NCoA-1 and NCoA-2, microinjection studies were performed, using the affinity-purified IgG's. Reporter genes were placed under the control of a minimal promoter containing either nuclear receptor or other response elements (Kamei et al., *supra*, 1996). Microinjection of anti-p/CIP IgG eliminated the ability of retinoic acid to activate an RAR-dependent transcription unit (Figure 4a), but was without effect on a promoter under the control of SP-1 elements or the CMV promoter. In similar experiments,

p/CIP also was required for the actions of estrogen, thyroid hormone and progesterone receptors (Figure 4b).

To determine whether depletion of CBP, rather than p/CIP itself, was responsible for the observed effects, the relative abilities of p/CIP, CBP, NCoA-1, and/or NCoA-2 to rescue the inhibitory effect of anti-p/CIP IgG was evaluated. No factor alone, including CBP, was able to rescue the inhibitory effect of anti-p/CIP IgG on RAR-dependent transcription, indicating that steric blockage or removal of CBP did not account for the observed effects. However, the simultaneous expression of both p/CIP and CBP fully restored retinoic acid transcriptional response in anti-p/CIP-treated cells (Figure 4c). These results indicate that both CBP and p/CIP are required together for nuclear receptor activation.

To independently confirm the need for p/CIP, the effect of a 137 amino acid region of p/CIP (aa 947-1084) containing the core CBP interaction domain was tested by microinjection assays. This peptide completely inhibited retinoic acid-dependent gene activation (Figure 4d; left), but did not block the activity of non-CBP-dependent promoters (Figure 4d; right).

The requirement of p/CIP for transcriptional activation by other CBP-dependent transcription factors, such as STAT also was examined (Bhattacharya et al., Nature 383:344-347 (1996); Zhang et al., Proc. Natl. Acad. Sci., *USA* 93:15092-15096 (1996); Horvai et al., Proc. Natl. Acad. Sci., *USA* 94:1074-1079 (1997)). The effect of anti-p/CIP and NCoA-1 IgG was evaluated by immunoinjection assay in cells, initially using interferon γ-dependent or TPA-dependent reporters. Anti-p/CIP IgG entirely inhibited the STAT-dependent and TPA-dependent transcriptional activation events (see

Figure 4e) and the inhibition was not restored by overexpression of CBP, alone. Independent confirmation was provided by over-expression of the CBP interaction domain of p/CIP (aa 947-1084), which blocked the ability of interferon γ or of TPA to stimulate transcriptional activation (Figure 4e). Further, C-terminally truncated CBP failed to enhance either interferon γ- or TPA-dependent transcription in cotransfection assays and could not rescue the block of retinoic acid- and interferon γ-dependent gene activation by injected anti-CBP IgG (Kamei et al., *supra*, 1996; Horvai et al., *supra*, 1997). These results indicate that p/CIP and CBP represent a functional complex, required for function by several CBP-dependent transcription factors in addition to nuclear receptors.

3. Roles of NCoA-1 and NCoA-2 in Nuclear Receptor Activation

Based on the requirement for p/CIP in both nuclear receptor and several CBP/p300-dependent transcription factors, it was important to evaluate the precise roles of NCoA-1/SRC-1 and NCoA-2/TIF-2, which, by cotransfection, enhance transactivation by nuclear receptors. Microinjection of anti-NCoA-1 IgG, but not of anti-NCoA-2 IgG, effectively inhibited retinoic acid-dependent transcription (Figure 5a), while these antisera failed to inhibit several control promoters that lacked nuclear receptor response elements (Figure 5a). In addition, anti-NCoA-1 IgG fully inhibited estrogen and thyroid hormone receptor stimulation (Figure 5b) and partially inhibited progesterone receptor stimulation (Figure 5b). Co-injection of NCoA-1, NCoA-2 or p/CIP expression vectors revealed that the inhibitory effects of anti-NCoA-1 IgG could be entirely reversed by either NCoA-1 or NCoA-2, but not by p/CIP (Figure 5c), consistent with a distinct role for this factor, and in

contrast to the requirement for both p/CIP and CBP to rescue the inhibitory actions of anti-p/CIP IgG. Co-injection of a CMV-CBP expression vector also failed to restore activity, consistent with the model that both NCoA-1 and the CBP/p300/p/CIP complex are independently required for nuclear receptor gene activation events (Figure 5c). In contrast, anti-NCoA-1 IgG exerted no effects on either cAMP- or interferon γ-dependent reporters (Figure 4f). These results indicate that NCoA-1 is selectively required as a coactivator for the ligand-activated nuclear receptor gene expression events; the requirement for the CBP/p300/p/CIP complex reflects a more general obligatory role in gene activation events.

4. Interaction Motifs of the Co-Integrator Complex Selectively Inhibit Transcriptional Effects of Distinct Signal Transactivation Pathways

In view of the relatedness of NCoA-1 and p/CIP, despite the apparent distinctions in their functional roles, and the involvement of CBP/p300 in activation of different classes of transcription factors, the ability of distinct interaction domains to selectively block the actions of specific signal transduction pathways at a nuclear level was examined. Delineation of the nuclear receptor interaction domains of p/CIP, NCoA-1, and NCoA-2 revealed the presence of highly conserved leucine, charged residue-rich domains (LCD's) that share a consensus core sequence, LXXLL (Figure 6a). This motif is found in both the nuclear receptor and the p/CIP interaction domains of CBP and in the CBP interaction domain of p/CIP.

Analysis of these putative interaction regions by the self-optimized prediction method (SOPM; Geourjon and Deleage, Protein Engineering 7:157-164 (1994), which is incorporated herein by reference) strongly suggested

that they represent helical domains, generally with amphipathic characteristics (Figure 6a). To begin to investigate whether these LCD's exert a critical interaction function, four amino acid mutations of this motif were introduced into the N-terminus of CBP (aa 65-76), abolishing interactions with nuclear receptors (Figure 6b). The minimal nuclear receptor interaction domain of NCoA-1 contains three such helical motifs, and a fourth such motif (LCD6) also is present in a variant of NCoA-1 (Onate et al., Science 270:1354-1357 (1995); Kamei et al., *supra*, 1997).

To assess the importance of these motifs in NCoA-1, a smaller region lacking helical domain 3 resulted in little or no decrease in binding to either estrogen or retinoic acid receptors, while deletion of helical domain 1 exhibited a small but significant decrease (Figure 6c). In contrast, a four amino acid substitution in the second NCoA-1 helical domain (LCD2; HRLL→AAAA), which would alter the properties of this helix, abolished interaction with both estrogen and retinoic acid receptors. Conversely, a 37 amino acid region of NCoA-1 containing LCD2, or 59 amino acids containing LCD6, was sufficient for binding to liganded nuclear receptors (Figure 6c, left). The addition of an excess 24 mer oligopeptide encompassing LCD2 effectively blocked interactions between liganded RAR and NCoA-1 *in vitro;* whereas a peptide corresponding to LCD1 was less effective. These results indicate that specific motifs can be both required and, in certain instances, sufficient, for interaction.

To assess the potential selective functional requirements of these helical motif sequences in the nuclear receptor interaction domain of NCoA-1, mutations in helical domains 2 or 3 were generated in the context of the holoprotein and tested for the ability to rescue

anti-NCoA-1 IgG inhibition of retinoic acid receptor function. Whereas wild-type NCoA-1 fully rescued activation, an NCoA-1 holoprotein harboring clustered point mutations in helical domain 3 (LCD3-mut) was completely ineffective at rescuing retinoic acid receptor function. NCoA-1 containing a helical domain 2 (LCD2-mut) mutation retained some residual efficacy (Figure 6d), consistent with the residual ability of the helical domains to mediate nuclear receptor interactions. Surprisingly, however, NCoA-1 harboring the helical domain 3 mutation (LCD3-mut) retained full functional ability in estrogen receptor-dependent gene activation, whereas LCD2-mut was completely ineffective at rescuing estrogen receptor function (Figure 6e). These results indicate that the helical interaction motifs of NCoA-1 afford a level of receptor specificity.

To independently assess the importance of these motifs, corresponding peptides were tested for their ability to inhibit specific activation events. NCoA-1 harbors two additional related helical interaction motifs, and a peptide encompassing one of these motifs (LCD4) can block nuclear receptor transcription factor function and does not impair STAT function (Figure 7a). Furthermore, a mutation within this motif markedly impairs the function of this region of p/CIP (data not shown). Thus, specific signal transduction pathways can be selectively blocked by distinct helical interaction motifs.

Other motifs, not required for nuclear receptor activation, also were examined to determine if, similarly, they are critical for coactivator function for other classes of CBP-dependent transcription factors, thus providing a means to selective block distinct signal transduction pathways. This study was initiated based on the demonstration that a critical STAT interaction domain

is found within the first 100 amino acids of CBP by coimmunoprecipitation (Horvai et al., *supra*, 1997). To determine whether a sequence of the CBP N-terminal 100 amino acids, distinct from the nuclear receptor motif, can both mediate interactions with STAT-1 and be required for STAT function, the effects of peptides corresponding to N-terminal regions of CBP on STAT-1 or retinoic acid receptor function was evaluated. Remarkably, a synthetic peptide against the N-terminal 22 amino acids of CBP (CBP N'P1; Figure 7b) markedly inhibited interferon γ-dependent gene activation, but was without effect on retinoic acid receptor function. The identical peptide, from which the N-terminal seven amino acids (MAENLLY) were deleted, abolished this effect (CBP N'-P2; Figure 7b), indicating that this sequence encompassed a motif required for STAT interaction and function. These results further support the functional significance of the STAT-1 interaction motif previously identified in the CBP N-terminus (Horvai et al., *supra*, 1997).

In parallel, the ability of the CBP N-terminal peptide to selectively block the inhibitory effects of STAT-1 or retinoic acid receptor-dependent transcription was examined by evaluating its effects on simultaneous stimulation by interferon γ and retinoic acid. The simultaneous addition of retinoic acid and interferon γ led to reciprocal inhibition of retinoic acid- and interferon-dependent reporter gene expression (Figure 7c). However, the addition of the CBP N-terminal 22 amino acid peptide (CBP N'-P1) relieved inhibition of RAR-dependent transcription by interferon γ, consistent with the hypothesis that this inhibitory effect represents, at least in part, competition for CBP coactivator complexes, analogous to that proposed for AP-1 and nuclear receptors (Kamei et al., *supra*, 1997). Together, these results are consistent with the hypothesis that different motifs are used in assembling

CBP-dependent complexes by different classes of transcription factors, and that peptides based on these motifs can selectively block specific signal transduction pathways.

The results disclosed herein indicate that p/CIP, which is associated with CBP/p300 in cell, is involved in regulating transcription by nuclear receptors and by other CBP-dependent factors, including STAT and AP-1. Furthermore, both the CBP/p/CIP complex and NCoA-1 are required to permit full ligand-activated gene transcription in the cells examined, while NCoA-1/SRC-1 is not required for other CBP-dependent transcription. Because CBP is capable of associating with a large number of additional factors, including myb, YY1, SREBP, myoD and the HLH1 factors, it is likely that p/CIP and CBP are components of a larger complex inportant for integration of many signal transduction pathways.

Studies have shown that the N-terminus of CBP alone is sufficient to potentiate CREB function using transient cotransfection assays (Bisotto et al., J. Biol. Chem. 271:17746-17750 (1996); Swope et al., J. Biol. Chem. 271:28138-28145 (1996)). In contrast, a recent study has shown that the C-terminus also was required in *in vitro* transcription assays (Nakajima et al., Genes and Devel. 11:738-747 (1997)). While not wishing to be bound by the following, the results disclosed herein indicate that conformational alterations in CBP holoprotein, which may be contributed by p/CIP, can modulate interactions with transcription factors and associated regulatory proteins, including protein kinases and those that have been shown to possess histone acetylase functions. Furthermore, p/CAF is capable of interacting with NCoA-1 as well as CBP41, although its role in mediating the transcriptional activation by nuclear receptors is unclear.

The nuclear receptor and CBP interaction domains within NCoA-1, NCoA-2, and p/CIP contain putative helical domains, referred to as LCD's, that are required and, in at least in some cases, sufficient, for receptor-specific interactions. Thus, the third helical domain in the nuclear receptor interaction domain of NCoA-1 is differentially utilized, being important for retinoic acid function, but not for estrogen receptor-dependent gene activation events. Similar LCD's are present in CBP and in other factors, including TIF-1 and RIP 140, as well as in the N-terminal interaction domain of p/CAF. Thus, many factors can have the ability to associate with the complexes formed on receptor homodimers or heterodimers bound to their cognate DNA site and contribute to the specificity of nuclear receptor pathways. Such an assembly of specific complexes of proteins based on these interaction motifs can provide a basis for receptor-specific and regulated aspects of nuclear receptor function.

As disclosed herein, helical interaction domains in CBP/p/CIP/NCoA proteins and other nuclear receptor interacting factors permitted the use of such domains to selectively block gene activation events in response to specific signal transduction pathways. Thus, peptides corresponding to CBP interaction domains selectively block nuclear receptor or STAT-1 function. The actions of specific inhibitory peptides indicates that partitioning of CBP accounts, at least in part, for trans-repression of nuclear receptor, STAT and AP-1 pathways.

What is claimed is:

1. A substantially purified nucleic acid molecule, comprising a nucleotide sequence encoding a p/CIP polypeptide.

2. The substantially purified nucleic acid molecule of claim 1, wherein said nucleotide sequence encodes substantially the same amino acid sequence as a p/CIP polypeptide having the amino acid sequence shown in Figure 1.

3. The substantially purified nucleic acid molecule of claim 2, wherein said nucleotide sequence is the nucleotide sequence shown in Figure 1.

4. A substantially purified nucleic acid molecule encoding an active fragment of a p/CIP polypeptide, comprising a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a p/CIP polypeptide.

5. The substantially purified nucleic acid molecule of claim 4, said active fragment comprising a CBP interaction domain.

6. The substantially purified nucleic acid molecule of claim 5, comprising a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a p/CIP polypeptide selected from the group consisting of about amino acids 758 to 1115 of p/CIP; about amino acids 947 to 1084 of p/CIP; and about amino acids 163 to 610 of p/CIP shown in Figure 1.

7. The substantially purified nucleic acid molecule of claim 4, said active fragment comprising a nuclear receptor interaction domain.

8. The substantially purified nucleic acid molecule of claim 7, comprising a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a p/CIP polypeptide selected from the group consisting of about amino acids 591 to 803 and about amino acids 680 to 740 of p/CIP shown in Figure 1.

9. A substantially purified p/CIP nucleotide sequence, comprising at least about 14 consecutive nucleotides of the nucleotide sequence shown in Figure 1, or a nucleotide sequence complementary thereto.

10. A substantially purified nucleic acid molecule, comprising a nucleotide sequence encoding a NCoA-2 polypeptide having substantially the same amino acid sequence as the amino acid sequence shown in Figure 2a.

11. The substantially purified nucleic acid molecule of claim 10, wherein said nucleotide sequence is the nucleotide sequence shown in Figure 2a.

12. A substantially purified nucleic acid molecule encoding an active fragment of a NCoA-2 polypeptide, comprising a nucleotide sequence encoding substantially the same amino acid sequence as a portion of a NCoA-2 polypeptide.

13. The substantially purified nucleic acid molecule of claim 12, said active fragment comprising a nuclear receptor interaction domain.

14. The substantially purified nucleic acid molecule of claim 13, comprising a nucleotide sequence encoding substantially the same amino acid sequence as about amino acids 562 to 808 of NCoA-2 shown in Figure 2a.

15. A substantially purified p/CIP polypeptide.

16. The substantially purified p/CIP polypeptide of claim 15, comprising substantially the same amino acid sequence as a p/CIP polypeptide having the amino acid sequence shown in Figure 1.

17. A substantially purified p/CIP active fragment, comprising substantially the same amino acid sequence as a portion of a p/CIP polypeptide.

18. The substantially purified p/CIP active fragment of claim 17, said active fragment comprising a CBP interaction domain.

19. The substantially purified p/CIP active fragment of claim 18, comprising substantially the same amino acid sequence as a portion of a p/CIP polypeptide selected from the group consisting of about amino acids 758 to 1115 of p/CIP; about amino acids 947 to 1084 of p/CIP; and about amino acids 163 to 610 of p/CIP shown in Figure 1.

20. The substantially purified p/CIP active fragment of claim 17, said active fragment comprising a nuclear receptor interaction domain.

21. The substantially purified p/CIP active fragment of claim 20, comprising substantially the same amino acid sequence as a portion of a p/CIP polypeptide selected from the group consisting of about amino acids 591 to 803 and about amino acids 680 to 740 of p/CIP shown in Figure 1.

22. A substantially purified NCoA-2 polypeptide, comprising substantially the same amino acid sequence as a NCoA-2 polypeptide having the amino acid sequence shown in Figure 2a.

23. A substantially purified NCoA-2 active fragment, comprising substantially the same amino acid sequence as a portion of a NCoA-2 polypeptide.

24. The substantially purified NCoA-2 active fragment of claim 23, said active fragment comprising a nuclear receptor interaction domain.

25. The substantially purified NCoA-2 active fragment of claim 24, comprising substantially the same amino acid sequence as about amino acids 562 to 808 of NCoA-2 shown in Figure 2a.

26. A method of identifying an effective agent that alters the association of a p/CIP polypeptide with a second protein, comprising the steps of:

(a) contacting a p/CIP polypeptide and a second protein with an agent under conditions that allow said p/CIP polypeptide to associate with said second protein; and

(b) detecting an altered association of said p/CIP polypeptide and said second protein.

27. The method of claim 26, wherein said p/CIP polypeptide has the amino acid sequence shown in Figure 1.

28. The method of claim 26, wherein said second protein is selected from the group consisting of a CBP protein, a nuclear receptor and a CBP/p300-dependent transcription factor.

29. The method of claim 26, wherein said altered association is detected by measuring the transcriptional activity of a reporter gene.

30. The method of claim 26, wherein said contacting is *in vitro*.

31. The method of claim 26, wherein said contacting is in a cell selected from the group consisting of a mammalian cell and a yeast cell.

32. A method of identifying a ligand for a nuclear receptor, comprising the steps of:

(a) contacting a NCoA polypeptide, or nuclear receptor interaction domain thereof, and a nuclear receptor with an agent under conditions that allow said NCoA polypeptide or nuclear receptor interaction domain thereof to associate with said nuclear receptor; and

(b) detecting an altered association between said NCoA polypeptide, or nuclear receptor binding fragment thereof, and said nuclear receptor,

where an increased association indicates that said agent is an agonistic ligand of said nuclear receptor and

where a decreased association indicates that said agent is an antagonistic ligand of said nuclear receptor.

```
    GGCGGCGAACGGATCAAAAGAATTTGCTGAACAGTGGACTCCGAGATCGGTAAAACGAAC
  1 ---------+---------+---------+---------+---------+---------+ 60(20)
    CCGCCGCTTGCCTAGTTTTCTTAAACGACTTGTCACCTGAGGCTCTAGCCATTTTGCTTG

    TCTTCCCTGCCCTTCCTGAACAGCTGTCAGTTGCTGATCTGTGATCAGGATGAGTGGACT
 61 ---------+---------+---------+---------+---------+---------+ 120(40)
    AGAAGGGACGGGAAGGACTTGTCGACAGTCAACGACTAGACACTAGTCCTACTCACCTGA
                                                  M  S  G  L  -

    AGGCGAAAGCTCTTTGGATCCGCTGGCCGCTGAGTCTCGGAAACGCAAACTGCCCTGTGA
121 ---------+---------+---------+---------+---------+---------+ 180
    TCCGCTTTCGAGAAACCTAGGCGACCGGCGACTCAGAGCCTTTGCGTTTGACGGGACACT
     G  E  S  S  L  D  P  L  A  A  E  S  R  K  L  K  L  P  C  D

    TGCCCCAGGACAGGGGCTTGTCTACAGTGGTGAGAAGTGGCGACGGGAGCAGGAGAGCAA
181 ---------+---------+---------+---------+---------+---------+ 240
    ACGGGGTCCTGTCCCCGAACAGATGTCACCACTCTTCACCGCTGCCCTCGTCCTCTCGTT
     A  P  G  Q  G  L  V  Y  S  G  E  K  W  R  R  E  Q  E  S  K  -

    GTACATAGAGGAGCTGGCAGAGCTCATCTCTGCAAATCTCAGCGACATCGACAACTTCAA
241 ---------+---------+---------+---------+---------+---------+ 300
    CATGTATCTCCTCGACCGTCTCGAGTAGAGACGTTTAGAGTCGCTGTAGCTGTTGAAGTT
```

FIG. 1A

```
    TGTCAAGCCAGATAAATGTGCCATCCTAAAGGAGACAGTGAGACAGATACGGCAAATAAA
301 ---------+---------+---------+---------+---------+---------+ 360
    ACAGTTCGGTCTATTTACACGGTAGGATTTCCTCTGTCACTCTGTCTATGCCGTTTATTT
     V  K  P  D  K  C  A  I  L  K  E  T  V  R  Q  I  R  Q  I  K  -

    AGAACAAGGAAAAACTATTTCCAGTGATGATGATGTTCAAAAAGCTGATGTGTCTTCTAC
361 ---------+---------+---------+---------+---------+---------+ 420
    TCTTGTTCCTTTTTGATAAAGGTCACTACTACTACAAGTTTTTCGACTACACAGAAGATG
     E  Q  G  K  T  I  S  S  D  D  D  V  Q  K  A  D  V  S  S  T  -

    AGGGCAGGGAGTCATTGATAAAGACTCTTTAGGACCGCTTTTACTACAGGCAGTGGATGG
421 ---------+---------+---------+---------+---------+---------+ 480
    TCCCGTCCCTCAGTAACTATTTCTGAGAAATCCTGGCGAAAATGATGTCCGTGACCTACC
     G  Q  G  V  I  D  K  D  S  L  G  P  L  L  L  Q  A  L  D  G  -

    TTTCCTGTTTGTGGTGAATCGAGATGGAAACATTGTATTCGTGTCAGAAAATGTCACACA
481 ---------+---------+---------+---------+---------+---------+ 540
    AAAGGACAAACACCACTTAGCTCTACCTTTGTAACATAAGCACAGTCTTTTACAGTGTGT
     F  L  F  V  V  N  R  D  G  N  I  V  F  V  S  E  N  V  T  Q  -

    GTATCTGCAGTACAAGCAGGAGGACCTGGTTAACACAAGTGTCTACAGCATCTTACATGA
541 ---------+---------+---------+---------+---------+---------+ 600
    CATAGACGTCATGTTCGTCCTCCTGGACCAATTGTGTTCACAGATGTCGTAGAATGTACT
     Y  L  Q  Y  K  Q  E  D  L  V  N  T  S  V  V  S  T  L  H  E  -

    GCCAAGACGGAAGGATTTCTTAAACACTTACCAAAATCCACAGTTAATGGAGTTTCTTGG
601 ---------+---------+---------+---------+---------+---------+ 660
    CGGTTCTGCCTTCCTAAAGAATTTGTGAATGGTTTTAGGTGTCAATTACCTCAAAGAACC
     P  R  R  K  D  F  L  N  T  Y  Q  N  P  Q  L  M  E  F  L  G  -

    ACTAATGAGAACCAGAGACAAAAAAGCCCCATACATTTTAATTGTCCGTATGTTGATGAA
661 ---------+---------+---------+---------+---------+---------+ 720
```

FIG. 1B

```
     L  M  R  T  R  D  K  K  A  P  Y  I  L  I  V  R  M  L  M  K  -

     AACACACGACATTTTGGAAGACGTGAATGCCAGTCCCGAAACGCGCCAGAGATATGAAAC
721  ---------+---------+---------+---------+---------+---------+ 780
     TTGTGTGCTGTAAAACCTTCTGCACTTACGGTCAGGGCTTTGCGCGGTCTCTATACTTTG
      T  H  D  I  L  E  D  V  N  A  S  P  E  T  R  Q  R  Y  E  T  -

     AATGCAGTGCTTTGCCCTGTCTCAGCCTCGCGCTATGCTGGAAGAAGGAGAAGACTTGCA
781  ---------+---------+---------+---------+---------+---------+ 840
     TTACGTCACGAAACGGGACAGAGTCGGAGCGCGATACGACCTTCTTCCTCTTCTGAACGT
      M  Q  C  F  A  L  S  Q  P  R  A  M  L  E  E  G  E  D  L  Q  -

     GTGCTGTATGATCTGCGTGGCTCGCCGCGTGACTGCGCCATTCCCATCCAGTCCCGAGAG
841  ---------+---------+---------+---------+---------+---------+ 900
     CACGACATACTAGACGCACCGAGCGGCGCACTGACGCGGTAAGGGTAGGTCAGGGCTCTC
      C  C  M  I  C  V  A  R  R  V  T  A  P  F  P  S  S  P  E  S  -

     CTTTATTACCAGACATGACCTTTCCGGAAAGGTTGTCAATATAGATACAAACTCACTTAG
901  ---------+---------+---------+---------+---------+---------+ 960
     GAAATAATGGTCTGTACTGGAAAGGCCTTTCCAACAGTTATATCTATGTTTGAGTGAATC
      F  I  T  R  H  D  L  S  G  K  V  V  N  I  D  T  N  S  L  R  -

     ATCTTCCATGAGGCCTGGCTTTGAAGACATAATCCGAAGATGTATCCAGAGGTTCTTCAG
961  ---------+---------+---------+---------+---------+---------+ 1020
     TAGAAGGTACTCCGGACCGAAACTTCTGTATTAGGCTTCTACATAGGTCTCCAAGAAGTC
      S  S  M  R  P  G  F  E  D  I  I  R  R  C  I  Q  R  F  F  S  -

     TCTGAATGATGGGCACTCATGGTCCCAGAAGCGTCACTATCAAGAAGCTTATGTTCATGG
1021 ---------+---------+---------+---------+---------+---------+ 1080
     AGACTTACTACCCGTCAGTACCAGGGTCTTCGCAGTGATAGTTCTTCGAATACAAGTACC
```

FIG. 1C

```
      L  N  D  G  Q  S  W  S  Q  K  R  H  Y  Q  E  A  Y  V  H  G  -

      CCACGCAGAGACCCCCGTGTATCGTTTCTCCTTGGCTGATGGAACTATTGTGAGTGCGCA
1081  ---------+---------+---------+---------+---------+---------+ 1140
      GGTGCGTCTCTGGGGGCACATAGCAAAGAGGAACCGACTACCTTGATAACACTCACGCGT
       H  A  E  T  P  V  Y  R  F  S  L  A  D  G  T  I  V  S  A  Q  -

      GACAAAAAGCAAACTCTTCCGCAATCCTGTAACGAATGATCGTCACGGCTTCATCTCGAC
1141  ---------+---------+---------+---------+---------+---------+ 1200
      CTGTTTTTCGTTTGAGAAGGCGTTAGGACATTGCTTACTAGCAGTGCCGAAGTAGAGCTG
       T  K  S  K  L  F  R  N  P  V  T  N  D  R  H  G  F  I  S  T  -

      CCACTTTCTTCAGAGAGAACAGAATGGATACAGACCAAACCCAATCCCGCAGGACAAAGG
1201  ---------+---------+---------+---------+---------+---------+ 1260
      GGTGAAAGAAGTCTCTCTTGTCTTACCTATGTCTGGTTTGGGTTAGGGCGTCCTGTTTCC
       H  F  L  Q  R  E  Q  N  G  Y  R  P  N  P  I  P  Q  D  K  G  -

      CATCCGACCTCCTGCAGCAGGGTGTGGCGTGAGCATGTCTCCAAATCAGAATGTACAGAT
1261  ---------+---------+---------+---------+---------+---------+ 1320
      GTAGGCTGGAGGACGTCGTCCCACACCGCACTCGTACAGAGGTTTAGTCTTACATGTCTA
       I  R  P  P  A  A  G  C  G  V  S  M  S  P  N  Q  N  V  Q  M  -

      GATGGGCAGCCGGACCTATGGCGTGCCAGACCCCAGCAACACAGGGCAGATGGGTGGAGC
1321  ---------+---------+---------+---------+---------+---------+ 1380
      CTACCCGTCGGCCTGGATACCGCACGGTCTGGGGTCGTTGTGTCCCGTCTACCCACCTCG
       M  G  S  R  T  Y  G  V  P  D  P  S  N  T  G  Q  M  G  G  A  -
```

FIG. 1D

```
     TAGGTACGGGGCTTCTAGTAGCGTAGCCTCACTGACGCCAGGACAAAGCCTACAGTCGCC
1381 ---------+---------+---------+---------+---------+---------+ 1440
     ATCCASTGCCCCGAAGATCATCGATCGGAGTGACTGCGGTCCTGTTTCGGATGTCAGCGG
      R  Y  G  A  S  S  S  V  A  S  L  T  P  G  Q  S  L  Q  S  P  -

     ATCTTCCTATCAGAACAGCAGCTATGGGCTCAGCATGAGCAGTCCCCCCCACGGCAGTCC
1441 ---------+---------+---------+---------+---------+---------+ 1500
     TAGAAGGATAGTCTTGTCGTCGATACCCGAGTCGTACTCGTCAGGGGGGGTGCCGTCAGC
      S  S  Y  Q  N  S  S  Y  G  L  S  M  S  S  P  P  H  G  S  P  -

     TGGTCTTGGCCCCAACCAGCAGAACATCATGATTTCCCCTCGGAATCGTGGCAGCCCAAA
1501 ---------+---------+---------+---------+---------+---------+ 1560
     ACCAGAACCGGGGTTGGTCGTCTTGTAGTACTAAAGGGGAGCCTTAGCACCGTCGGGTTT
      G  L  G  P  N  Q  Q  N  I  M  I  S  P  R  N  R  G  S  P  K  -

     GATGGCCTCCCACCAGTTCTCTCCTGCTGCAGGTGCACACTCACCCATGGGACCTTCTGG
1561 ---------+---------+---------+---------+---------+---------+ 1620
     CTACCGGAGGGTGGTCAAGAGAGGACGACGTCCACGTGTGAGTGGGTACCCTGGAAGACC
      M  A  S  H  Q  F  S  P  A  A  G  A  H  S  P  M  G  P  S  G  -
     CAACACAGGGAGCCACAGCTTTTCTAGCAGCTCCCTCAGTGCCTTGCAAGCCATCAGTGA
1621 ---------+---------+---------+---------+---------+---------+ 1680
     GTTGTGTCCCTCGGTGTCGAAAAGATCGTCGAGGGACTCACGGAACGTTCGGTAGTCACT
      N  T  G  S  H  S  F  S  S  S  S  L  S  A  L  Q  A  I  S  E  -
     AGGCGTGGGGACCTCTCTTTTATCTACTCTGTCCTCACCAGGCCCCAAACTGGATAATTC
1681 ---------+---------+---------+---------+---------+---------+ 1740
     TCCGCACCCCTGGAGAGAAAATAGATGAGACAGGAGTGGTCCGGGGTTTGACCTATTAAG
      G  V  G  T  S  L  L  S  T  L  S  S  P  G  P  K  L  D  N  S  -

     TCCCAATATGAATATAAGCCAGCCAAGTAAAGTGAGTGGTCAGGACTNTAAGAGCCCCCT
1741 ---------+---------+---------+---------+---------+---------+ 1800
     AGGGTTATACTTATATTCGGTCGGTTCATTTCACTCACCAGTCCTGANATTCTCGGGGGA
      P  N  M  N  I  S  Q  P  S  K  V  S  G  Q  D  ?  K  S  P  L  -
```

FIG. 1E

```
     AGGCTTATACTGTGAACAGAATCCAGTGGAGAGTTCAGTGTGTCAGTCAAACAGCAGAGA
1801 ---------+---------+---------+---------+---------+---------+ 1860
     TCCGAATATGACACTTGTCTTAGGTCACCTCTCAAGTCACACAGTCAGTTTGTCGTCTCT
      G  L  Y  C  E  Q  N  P  V  E  S  S  V  C  Q  S  N  S  R  D  -

     TCCCCAAGTGAAAAAAGAAAGCAAGGAGAGCAGTGGGGAGGTGTCAGAGACGCCCAGGGG
1861 ---------+---------+---------+---------+---------+---------+ 1920
     AGGGGTTCACTTTTTTCTTTCGTTCCTCTCGTCACCCCTCCACAGTCTCTGCGGGTCCCC
      P  Q  V  K  K  E  S  K  E  S  S  G  E  V  S  E  T  P  R  G  -

     ACCTCTGGAAAGCAAAGGCCACAAGAAACTGCTGCAGTTACTCACGTGCTCCTCCGACGA
1921 ---------+---------+---------+---------+---------+---------+ 1980
     TGGAGACCTTTCGTTTCCGGTGTTCTTTGACGACGTCAATGAGTGCACGAGGAGGCTGCT
      P  L  E  S  K  G  H  K  K  L  L  Q  L  L  T  C  S  S  D  D  -

     CCGAGGCCATTCCTCCTTGACCAACTCTCCCCTGGATCCAAACTGCAAAGACTCTTCCGT
1981 ---------+---------+---------+---------+---------+---------+ 2040
     GGCTCCGGTAAGGAGGAACTGGTTGAGAGGGGACCTAGGTTTGACGTTTCTGAGAAGGCA
      R  G  H  S  S  L  T  N  S  P  L  D  P  N  C  K  D  S  S  V  -

     TAGTGTCACCAGCCCCTCTGGAGTGTCCTCCTCAACATCAGGGACAGTGTGTTCCACCTC
2041 ---------+---------+---------+---------+---------+---------+ 2100
     ATCACAGTGGTCGGGGAGACCTCACAGGAGGAGTTGTAGTCCCTGTCACAGAAGGTGGAG
      S  V  T  S  P  S  G  V  S  S  S  T  S  G  T  V  S  S  T  S  -

     CAATGTGCATGGGTCTCTGTTGCAAGAGAAACACCGGATTTTGCACAAGTTGCTGCAGAA
2101 ---------+---------+---------+---------+---------+---------+ 2160
     GTTACACGTACCCAGAGACAACGTTCTCTTTGTGGCCTAAAACGTGTTCAACGACGTCTT
      N  V  H  G  S  L  L  Q  E  K  H  R  I  L  H  K  L  L  Q  N  -
```

FIG. 1F

```
     TGGCAACTCCCCAGCGGAGGTCGCCAAGATCACTGCAGAGGCCACTGGGAAGGACACGAG
2161 ---------+---------+---------+---------+---------+---------+ 2220
     ACCGTTGAGGGGTCGCCTCCAGCGGTTCTAGTGACGTCTCCGGTGACCCTTCCTGTGCTC
      G  N  S  P  A  E  V  A  K  I  T  A  E  A  T  G  K  D  T  S  -
     CAGCACTGCTTCCTGTGGAGAGGGGACAACCAGGCAGGAGCAGCTGAGTCCTAAGAAGAA
2221 ---------+---------+---------+---------+---------+---------+ 2280
     GTCGTGACGAAGGACACCTCTCCCCTGTTGGTCCGTCCTCGTCGACTCAGGATTCTTCTT
      S  T  A  S  C  G  E  G  T  T  R  Q  E  Q  L  S  P  K  K  K  -

     GGAGAATAATGCTCTGCTTAGATACCTGCTGGACAGGGATGACCCCAGTGATGTGCTTGC
2281 ---------+---------+---------+---------+---------+---------+ 2340
     CCTCTTATTACGAGACGAATCTATGGACGACCTGTCCCTACTGGGGTCACTACACGAACG
      E  N  N  A  L  L  R  Y  L  L  D  R  D  D  P  S  D  V  L  A  -
     CAAAGAGCTGCAGCCCCAGGCCGACAGTGGGGACAGTAAACTGAGTCAGTGCAGCTGCTC
2341 ---------+---------+---------+---------+---------+---------+ 2400
     GTTTCTCGACGTCGGGGTCCGGCTGTCACCCCTGTCATTTGACTCAGTCACGTCGACGAG
      K  E  L  Q  P  Q  A  D  S  G  D  S  K  L  S  Q  C  S  C  S  -

     CACCAATCCCAGCTCTGGCCAAGAGAAAGACCCCAAAATTAAGACCGAGACGAACGACGA
2401 ---------+---------+---------+---------+---------+---------+ 2460
     GTGGTTAGGGTCGAGACCGGTTCTCTTTCTGGGGTTTTAATTCTGGCTCTGCTTGCTGCT
      T  N  P  S  S  G  Q  E  K  D  P  K  I  K  T  E  T  N  D  E  -

     GGTATCGGGAGACCTGGATAATCTAGATGCCATTCTTGGAGATTTGACCAGTTCTGACTT
2461 ---------+---------+---------+---------+---------+---------+ 2520
     CCATAGCCCTCTGGACCTATTAGATCTACGGTAAGAACCTCTAAACTGGTCAAGACTGAA
      V  S  G  D  L  D  N  L  D  A  I  L  G  D  L  T  S  S  D  F  -

     CTACAACAATCCTACAAATGGCGGTCACCCAGGGGCCAAACAGCAGATGTTTGCAGGACC
2521 ---------+---------+---------+---------+---------+---------+ 2580
```

FIG. 1G

```
      Y  N  N  P  T  N  G  G  H  P  G  A  K  Q  Q  M  F  A  G  P  -

      GAGTTCTCTGGGTTTGCGAAGTCCACAGCCTGTGCAGTCTGTTCGTCCTCCATATAACCG
2581  ---------+---------+---------+---------+---------+---------+  2640
      CTCAAGAGACCCAAACGCTTCAGGTGTCGGACACGTCAGACAAGCAGGAGGTATATTGGC
       S  S  L  G  L  R  S  P  Q  P  V  Q  S  V  R  P  P  Y  N  R  -

      AGCGGTGTCTCTGGATAGCCCTGTGTCTGTTGGCTCAGGTCCGCCAGTGAAGAATGTCAG
2641  ---------+---------+---------+---------+---------+---------+  2700
      TCGCCACAGAGACCTATCGGGACACAGACAACCGAGTCCAGGCGGTCACTTCTTACAGTC
       A  V  S  L  D  S  P  V  S  V  G  S  G  P  P  V  K  N  V  S  -

      TGCTTTCCCTGGGTTACCAAAACAGCCCATACTGGCTGGGAATCCAAGAATGATGGATAG
2701  ---------+---------+---------+---------+---------+---------+  2760
      ACGAAAGGGACCCAATGGTTTTGTCGGGTATGACCGACCCTTAGGTTCTTACTACCTATC
       A  F  P  G  L  P  K  Q  P  I  L  A  G  N  P  R  M  M  D  S  -

      TCAGGAGAATTACGGTGCCAACATGGGCCCAAACAGAAATGTTCCTGTGAATCCGACTTC
2761  ---------+---------+---------+---------+---------+---------+  2820
      AGTCCTCTTAATGCCACGGTTGTACCCGGGTTTGTCTTTACAAGGACACTTAGGCTGAAG
       Q  E  N  Y  G  A  N  M  G  P  N  R  N  V  P  V  N  P  T  S  -

      CTCCCCCGGAGACTGGGGCTTAGCTAACTCAAGGGCCAGCAGAATGGAGCCTCTGGCATC
2821  ---------+---------+---------+---------+---------+---------+  2880
      GAGGGGGCCTCTGACCCCGAATCGATTGAGTTCCCGGTCGTCTTACCTCGGAGACCGTAG
       S  P  G  D  W  G  L  A  N  S  R  A  S  R  M  E  P  L  A  S  -
```

FIG. 1H

```
     AAGTCCCCTGGGAAGAACTGGAGCCGATTACAGTGCCACTTTACCCAGACCTGCCATGGG
2881 ---------+---------+---------+---------+---------+---------+ 2940
     TTCAGGGGACCCTTCTTGACCTCGGCTAATGTCACGGTGAAATGGGTCTGGACGGTACCC
      S  P  L  G  R  T  G  A  D  Y  S  A  T  L  P  R  P  A  M  G  -
     GGGCTCTGTGCCTACCTTGCCACTTCGTTCTAATCGACTGCCAGGTGCAAGACCATCGTT
2941 ---------+---------+---------+---------+---------+---------+ 3000
     CCCGAGACACGGATGGAACGGTGAAGCAAGATTAGCTGACGGTCCACGTTCTGGTAGCAA
      G  S  V  P  T  L  P  L  R  S  N  R  L  P  G  A  R  P  S  L  -

     GCAGCAACAGCAGCAGCAACAGCAGCAACAGCAACAACAACAGCAGCAACAGCAGCAGCA
3001 ---------+---------+---------+---------+---------+---------+ 3060
     CGTCGTTGTCGTCGTCGTTGTCGTCGTTGTCGTTGTTGTTGTCGTCGTTGTCGTCGTCGT
      Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  -

     ACAGCAGCAGATGCTTCAAATGAGAACTGGTGAGATTCCCATGGGAATGGGAGTCAATCC
3061 ---------+---------+---------+---------+---------+---------+ 3120
     TGTCGTCGTCTACGAAGTTTACTCTTGACCACTCTAAGGGTACCCTTACCCTCAGTTAGG
      Q  Q  Q  M  L  Q  M  R  T  G  E  I  P  M  G  M  G  V  N  P  -

     NTATAGCCCAGCAGTGCAGTCTAACCAACCAGGTTCCTGGCCAGAGGGCATGCTCTCTAT
3121 ---------+---------+---------+---------+---------+---------+ 3180
     NATATCGGGTCGTCACGTCAGATTGGTTGGTCCAAGGACCGGTCTCCCGTACGAGAGATA
      Y  S  P  A  V  Q  S  N  Q  P  G  S  W  P  E  G  M  L  S  M  -

     GGAACAAGGTCCTCACGGGTCTCAAAATAGGCCTCTTCTTAGAAACTCTCTGGATGATCT
3181 ---------+---------+---------+---------+---------+---------+ 3240
     CCTTGTTCCAGGAGTGCCCAGAGTTTTATCCGGAGAAGAATCTTTGAGAGACCTACTAGA
      E  Q  G  P  H  G  S  Q  N  R  P  L  L  R  N  S  L  D  D  L  -

     GCTTGGGCCACCTTCTAACGCAGAGGGCCAGAGTGACGAGAGAGCTCTGCTGGACCAGCT
3241 ---------+---------+---------+---------+---------+---------+ 3300
     CGAACCCGGTGGAAGATTGCGTCTCCCGGTCTCACTGCTCTCTCGAGACGACCTGGTCGA
```

FIG. 1I

```
     GCACACATTCCTGAGCAACACAGATGCCACAGGTCTGGAGGAGATCGACAGGGCCTTGGG
3301 ---------+---------+---------+---------+---------+---------+ 3360
     CGTGTGTAAGGACTCGTTGTGTCTACGGTGTCCAGACCTCCTCTAGCTGTCCCGGAACCC
      H  T  F  L  S  N  T  D  A  T  G  L  E  E  I  D  R  A  L  G  -

     AATTCCTGAGCTCGTGAATCAGGGACAAGCTTTGGAGTCCAAACAGGATGTTTTCCAAGG
3361 ---------+---------+---------+---------+---------+---------+ 3420
     TTAAGGACTCGAGCACTTAGTCCCTGTTCGAAACCTCAGGTTTGTCCTACAAAAGGTTCC
      I  P  E  L  V  N  Q  G  Q  A  L  E  S  K  Q  D  V  F  Q  G  -
     CCAAGAAGCAGCAGTAATGATGGATCAGAAGGCTGCACTATATGGACAGACATACCCAGC
3421 ---------+---------+---------+---------+---------+---------+ 3480
     GGTTCTTCGTCGTCATTACTACCTAGTCTTCCGACGTGATATACCTGTCTGTATGGGTCG
      Q  E  A  A  V  M  M  D  Q  K  A  A  L  Y  G  Q  T  Y  P  A  -
     TCAGGGTCCTCCCCTTCAAGGAGGCTTTAACCTTCAGGGACAGTCACCATCGTTTAACTC
3481 ---------+---------+---------+---------+---------+---------+ 3540
     AGTCCCAGGAGGGGAAGTTCCTCCGAAATTGGAAGTCCCTGTCAGTGGTAGCAAATTGAG
      Q  G  P  P  L  Q  G  G  F  N  L  Q  G  Q  S  P  S  F  N  S  -

     TATGATGGGTCAGATTAGCCAGCAAGGCAGCTTTCCTCTGCAAGGCATGCATCCTAGAGC
3541 ---------+---------+---------+---------+---------+---------+ 3600
     ATACTACCCAGTCTAATCGGTCGTTCCGTCGAAAGGAGACGTTCCGTACGTAGGATCTCG
      M  M  G  Q  I  S  Q  Q  G  S  F  P  L  Q  G  M  H  P  R  A  -

     CGGCCTCGTGAGACCAAGGACCAACACCCCGAAGCAGCTGAGAATGCAGCTTCAGCAGAG
3601 ---------+---------+---------+---------+---------+---------+ 3660
     GCCGGAGCACTCTGGTTCCTGGTTGTGGGGCTTCGTCGACTCTTACGTCGAAGTCGTCTC
      G  L  V  R  P  R  T  N  T  P  K  Q  L  R  M  Q  L  Q  Q  R  -
```

FIG. 1J

```
     GCTACAGGGCCAGCAGTTTTTAAATCAGAGCCGGCAGGCACTTGAAATGAAAATGGAGAA
3661 ---------+---------+---------+---------+---------+---------+ 3720
     CGATGTCCCGGTCGTCAAAAATTTAGTCTCGGCCGTCCGTGAACTTTACTTTTACCTCTT
      L  Q  G  Q  Q  F  L  N  Q  S  R  Q  A  L  E  M  K  M  E  N  -

     CCCTGCTGGCACTGCTGTGATGAGGCCCATGATGCCCCAGGCTTTCTTTAATGCCCAAAT
3721 ---------+---------+---------+---------+---------+---------+ 3780
     GGGACGACCGTGACGACACTACTCCGGGTACTACGGGGTCCGAAAGAAATTACGGGTTTA
      P  A  G  T  A  V  M  R  P  M  M  P  Q  A  F  F  N  A  Q  M  -
     GGCTGCCCAGCAGAAACGAGAGCTGATGAGCCATCACCTGCAGCAGCAGAGGATGGCGAT
3781 ---------+---------+---------+---------+---------+---------+ 3840
     CCGACGGGTCGTCTTTGCTCTCGACTACTGGGTAGTGGACGTCGTCGTCTCCTACCGCTA
      A  A  Q  Q  K  R  E  L  M  S  H  H  L  Q  Q  Q  R  M  A  M  -

     GATGATGTCACAACCACAGCCTCAGGCCTTCAGCCCACCTCCCAACGTCACCGCCTCCCC
3841 ---------+---------+---------+---------+---------+---------+ 3900
     CTACTACAGTGTTGGTGTCGGAGTCCGGAAGTCGGGTGGAGGGTTGCAGTGGCGGAGGGG
      M  M  S  Y  P  Y  P  Q  A  F  S  P  P  P  N  V  T  A  S  P  -
     CAGCATGGACGGGGTTTTGGCAGGTTCAGCAATGCCGCAAGCCCCTCCACAACAGTTTCC
3901 ---------+---------+---------+---------+---------+---------+
     GTCGTACCTGCCCCAAAACCGTCCAAGTCGTTACGGCGTTCGGGGAGGTGTTGTCAAAGG
      S  M  D  G  V  L  A  G  S  A  M  P  Q  A  P  P  Q  Q  F  P  -

     ATATCCAGCAAATTACGGAACGGGACAACCACCAGTAGCCAGCCTTTGGTCGAGGCTCGA
3961 ---------+---------+---------+---------+---------+---------+ 4020
     TATAGGTCGTTTAATGCCTTGCCCTGTTGGTGGTCATCGGTCGGAAACCAGCTCCGAGCT
      Y  P  A  N  Y  G  T  G  Q  P  P  V  A  S  L  W  S  R  L  E  -

     GTCCTCCCAGTGCAATGATGTCATCAAGAATGGGGCCTTCCCAGAATGCCATGGTGCAGC
4021 ---------+---------+---------+---------+---------+---------+ 4080
     CAGGAGGGTCACGTTACTACAGTAGTTCTTACCCCGGAAGGGTCTTACGGTACCACGTCG
      S  S  Q  C  N  D  V  I  K  N  G  A  F  P  E  C  H  G  A  A  -
```

FIG. 1K

```
     ATCCTCAGCCCACACCCATGTATCAGCCTTCAGATATGAAGGGGTGGCCGTCAGGGAACC
4081 ---------+---------+---------+---------+---------+---------+ 4140
     TAGGAGTCGGGTGTGGGTACATAGTCGGAAGTCTATACTTCCCCACCGGCAGTCCCTTGG
      S  S  A  H  T  H  V  S  A  F  R  Y  E  G  V  A  V  R  E  P  -
     TGGCCAGGAATGGCTCCTTCCCCCAGCAGCAGTTTGCTCCCCAGGGGAACCCTGCAGCCT
4141 ---------+---------+---------+---------+---------+---------+ 4200
     ACCGGTCCTTACCGAGGAAGGGGGTCGTCGTCAAACGAGGGGTCCCCTTGGGACGTCGGA
      G  Q  E  W  L  L  P  P  A  A  V  C  S  P  G  E  P  C  S  L  -

     ACAACATGGTGCATATGAACAGCAGCGGTGGGCACTTGGGACAGATGGCCATGACCCCCA
4201 ---------+---------+---------+---------+---------+---------+ 4260
     TGTTGTACCACGTATACTTGTCGTCGCCACCCGTGAACCCTGTCTACCGGTACTGGGGGT
      Q  H  G  A  Y  E  Q  Q  R  W  A  L  G  T  D  G  H  D  P  H  -

     TGCCCATGTCTGGCATGCCCATGGGCCCCGATCAGAAATACTGCTGACATCTCCCTAGTG
4261 ---------+---------+---------+---------+---------+---------+ 4320
     ACGGGTACAGACCGTACGGGTACCCGGGGCTAGTCTTTATGACGACTGTAGAGGGATCAC
      A  H  V  W  H  A  H  G  P  R  S  E  I  L  L  T  S  P  *

     GGACTGACTGTACAGATGACACTGCACAGGATCATCAGGACGTGGCGGCGAGTCATTGTC
4321 ---------+---------+---------+---------+---------+---------+ 4380
     CCTGACTGACATGTCTACTGTGACGTGTCCTAGTAGTCCTGCACCGCCGCTCAGTAACAG

     TAAGCATCCAGCTTGGAAGCAAGGCCAGCGTGACCAGCAGCGGGGTCTGTGCTGTCATTT
4381 ---------+---------+---------+---------+---------+---------+ 4440
     ATTCGTAGGTCGAACCTTCGTTCCGGTCGCACTGGTCGTCGCCCCAGACACGACAGTAAA

     GAGCAGAGCTGGGTCTCGCTGAAGCGCACTGTCTACCTGATGCCCTGCCTCTGTGTGGCA
4441 ---------+---------+---------+---------+---------+---------+ 4500
```

FIG. 1L

```
     AGGTGTTCTGCCTCATGAGGATGTGATTCTGGAGATGGGGTGTTCGTAAGCACCGCTCTC
4501 ---------+---------+---------+---------+---------+---------+ 4560
     TCCACAAGACGGAGTACTCCTACACTAAGACCTCTACCCCACAAGCATTCGTGGCGAGAG

     TTACGTCACTCCCTTCTGCCTCGCCAGCCAAAGTCTTCACGTAGATCTAGATGGCTAGGG
4561 ---------+---------+---------+---------+---------+---------+ 4620
     AATGCAGTGAGGGAAGACGGAGCGGTCGGTTTCAGAAGTGCATCTAGATCTACCGATCCC

     TTTCTGTCTTGCAGCACTGGACGAGGGGGCACACTCTGCCTTCTCGCGTGTCGTCAGCAA
4621 ---------+---------+---------+---------+---------+---------+ 4680
     AAAGACAGAACGTCGTGACCTGCTCCCCCGTGTCAGACGGAAGAGCGCACAGCAGTCGTT

     GTTAGTTCGTGTCGCTCTCCTGTCCAGTGCAATCAGTGTTTCTGCGCTCTTGTCCTTTAC
4681 ---------+---------+---------+---------+---------+---------+ 4740
     CAATCAAGCACAGCGAGAGGACAGGTCACGTTAGTCACAAAGACGCGAGAACAGGAAATG

     AGGTGTAATCCCCAAGTCTGTCGTCCTAGTCTCTCCTGGTGAAGTCCCCGTACCTGTAAT
4741 ---------+---------+---------+---------+---------+---------+ 4800
     TCCACATTAGGGGTTCAGACAGCAGGATCAGAGAGGACCACTTCAGGGGCATGGACATTA

     CTCAACAATTCTCATTGAAGTTTAAATGGCTTTTGAAAAAAGGGAAAAATGAAAATGGCA
4801 ---------+---------+---------+---------+---------+---------+
```

FIG. 1M

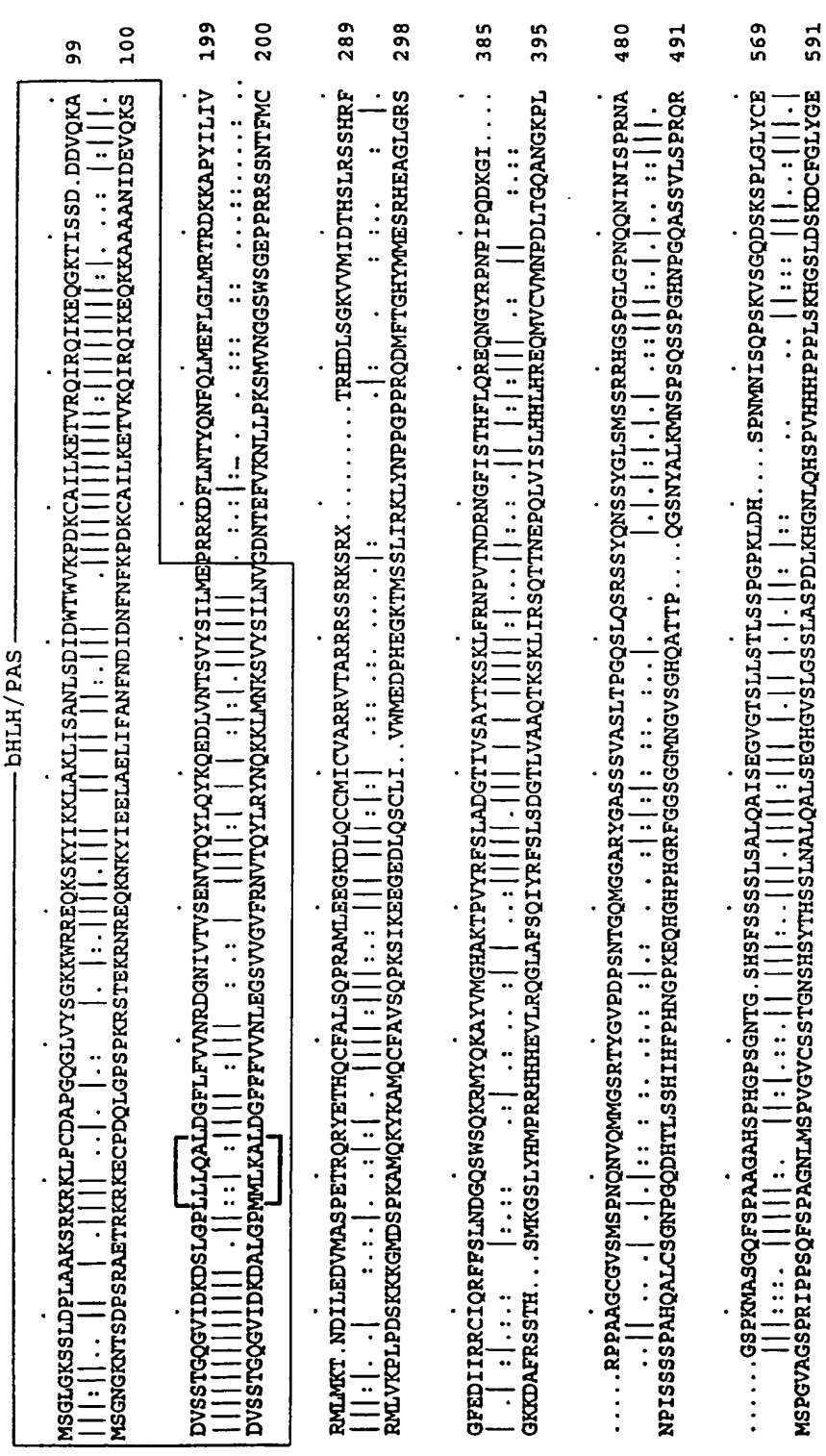

FIG. 2A-1

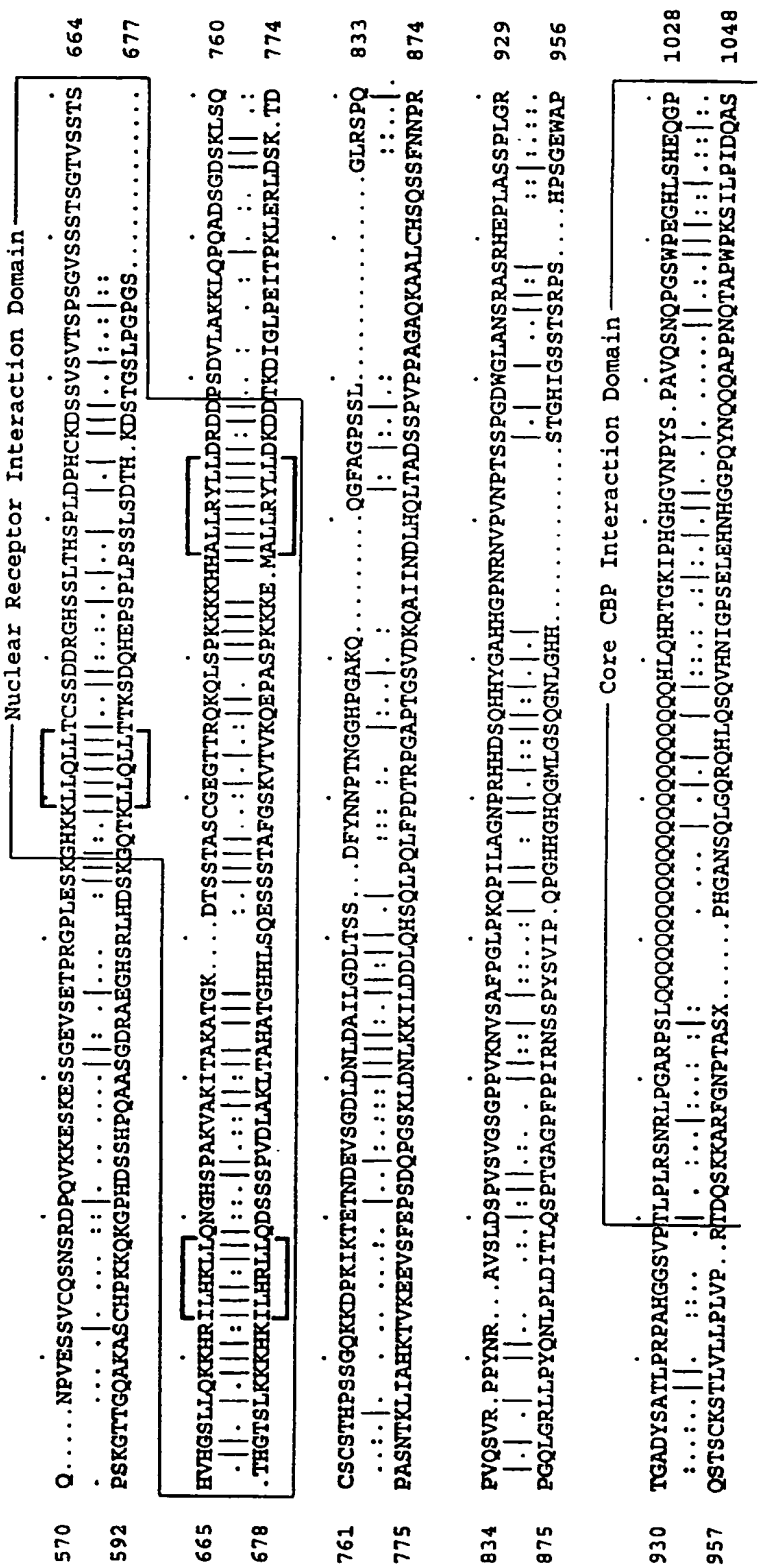
Nuclear Receptor Interaction Domain
Core CBP Interaction Domain

FIG. 2A-2

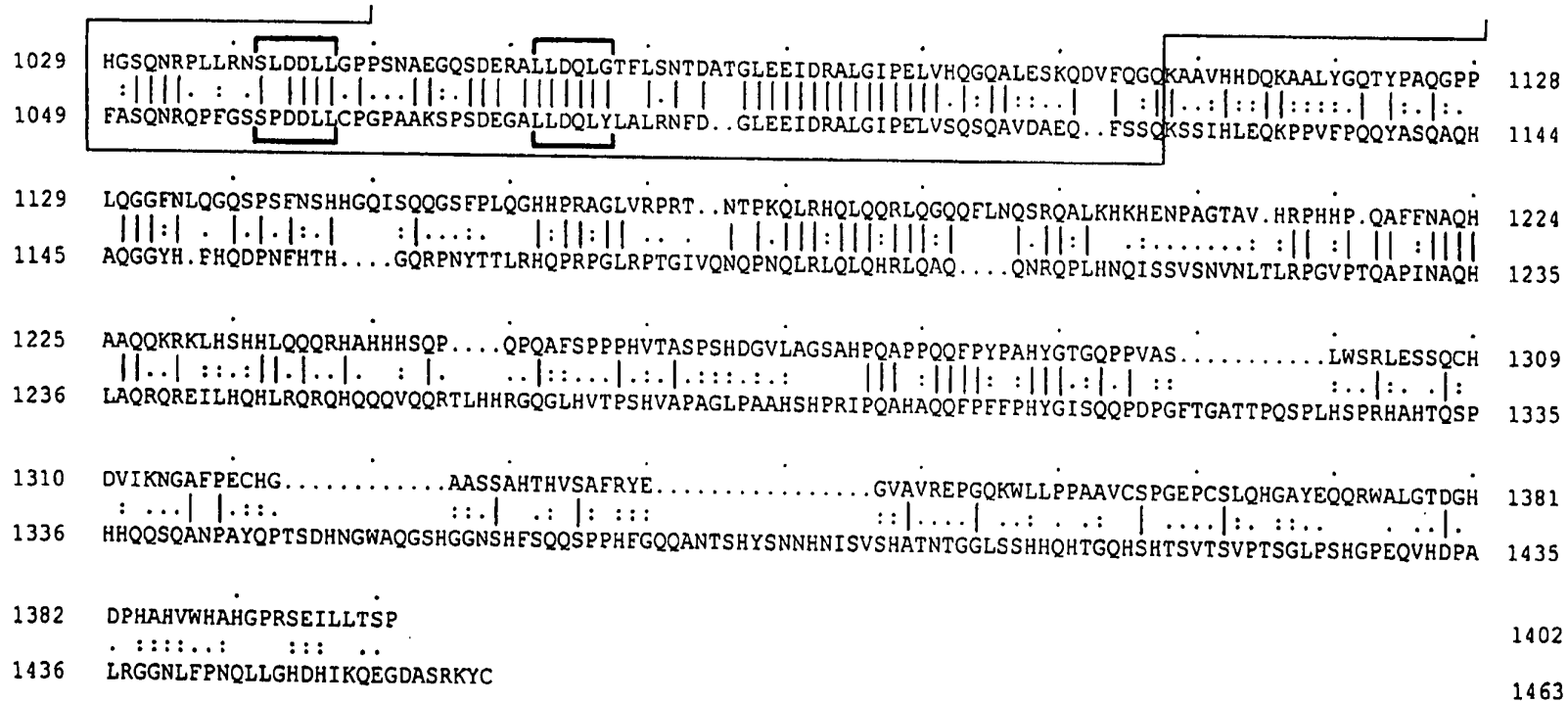
1029 HGSQNRPLLRNSLDDLLGPPSNAEGQSDERALLDQLGTFLSNTDATGLEEIDRALGIPELVHQGQALESKQDVFQGQKAAVHHDQKAALYGQTYPAQGPP 1128
1049 FASQNRQPFGSSPDDLLCPGPAAKSPSDEGALLDQLYLALRNFD..GLEEIDRALGIPELVSQSQAVDAEQ..FSSQKSSIHLEQKPPVFPQQYASQAQH 1144
1129 LQGGFNLQGQSPSFNSHHGQISQQGSFPLQGHHPRAGLVRPRT..NTPKQLRHQLQQRLQGQQFLNQSRQALKHKHENPAGTAV.HRPHHP.QAFFNAQH 1224
1145 AQGGYH.FHQDPNFHTH....GQRPNYTTLRHQPRPGLRPTGIVQNQPNQLRLQLQHRLQAQ....QNRQPLHNQISSVSNVNLTLRPGVPTQAPINAQH 1235
1225 AAQQKRKLHSHHLQQQRHAHHHSQP....QPQAFSPPPHVTASPSHDGVLAGSAHPQAPPQQFPYPAHYGTGQPPVAS.....LWSRLESSQCH 1309
1236 LAQRQREILHQHLRQRQHQQQVQQRTLHHRGQGLHVTPSHVAPAGLPAAHSHPRIPQAHAQQFPFFPHYGISQQPDPGFTGATTPQSPLHSPRHAHTQSP 1335
1310 DVIKNGAFPECHG.....AASSAHTHVSAFRYE.....GVAVREPGQKWLLPPAAVCSPGEPCSLQHGAYEQQRWALGTDGH 1381
1336 HHQQSQANPAYQPTSDHNGWAQGSHGGNSHFSQQSPPHFGQQANTSHYSNNHNISVSHATNTGGLSSHHQHTGQHSHTSVTSVPTSGLPSHGPEQVHDPA 1435
1382 DPHAHVWHAHGPRSEILLTSP 1402
1436 LRGGNLFPNQLLGHDHIKQEGDASRKYC 1463

FIG. 2A-3

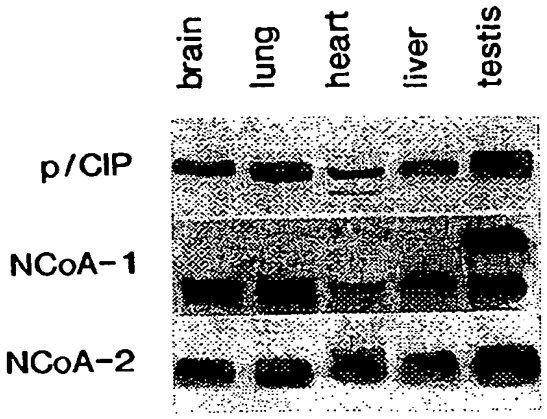

FIG. 2B

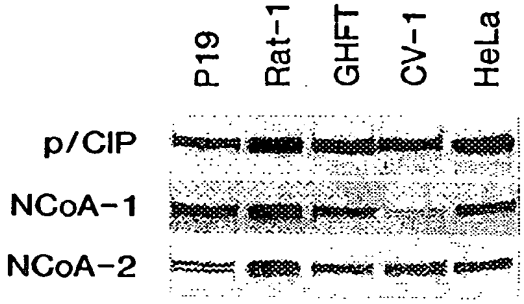

FIG. 2C

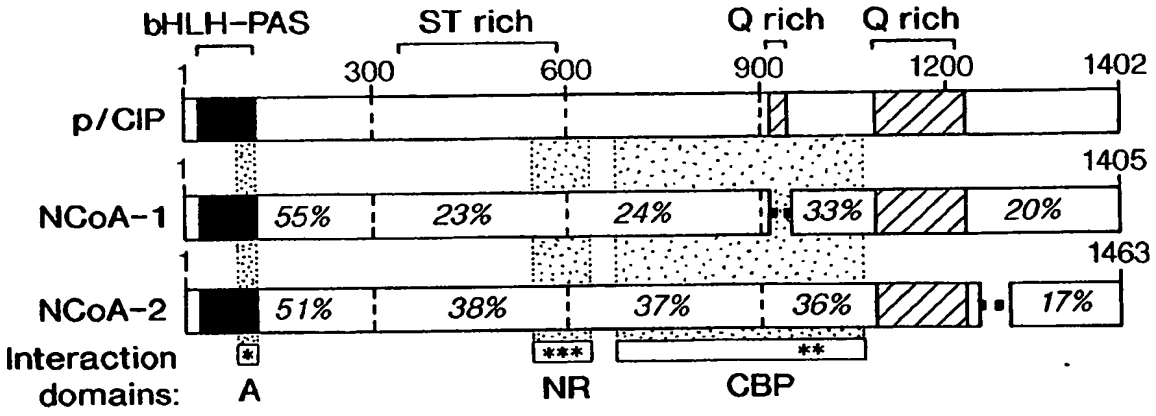

FIG. 2D

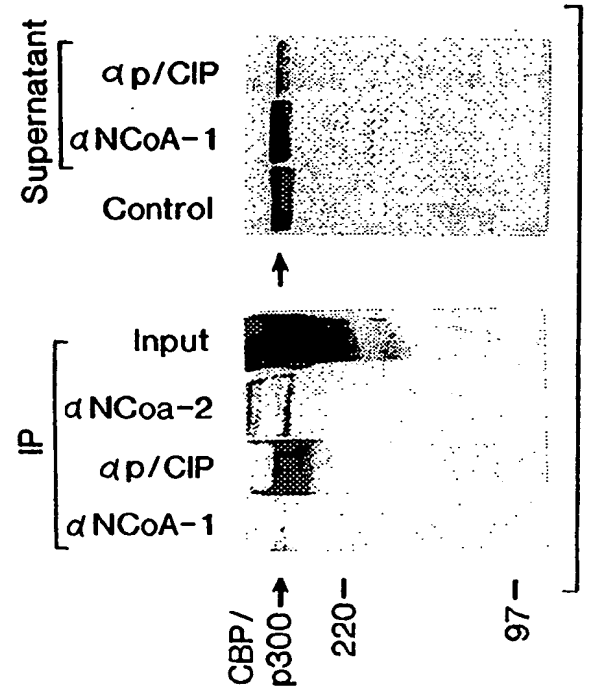

FIG. 3B

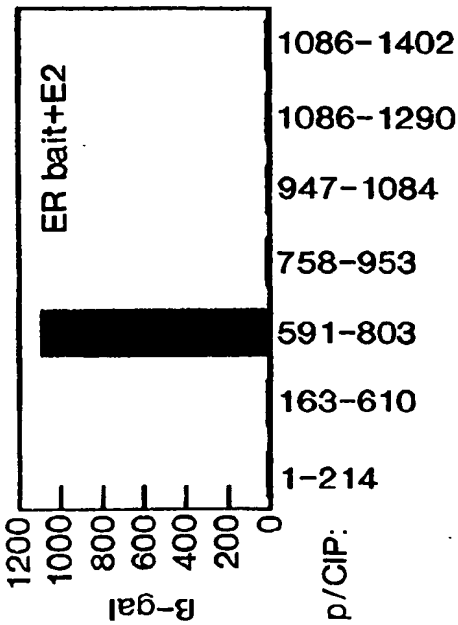

FIG. 3C-2

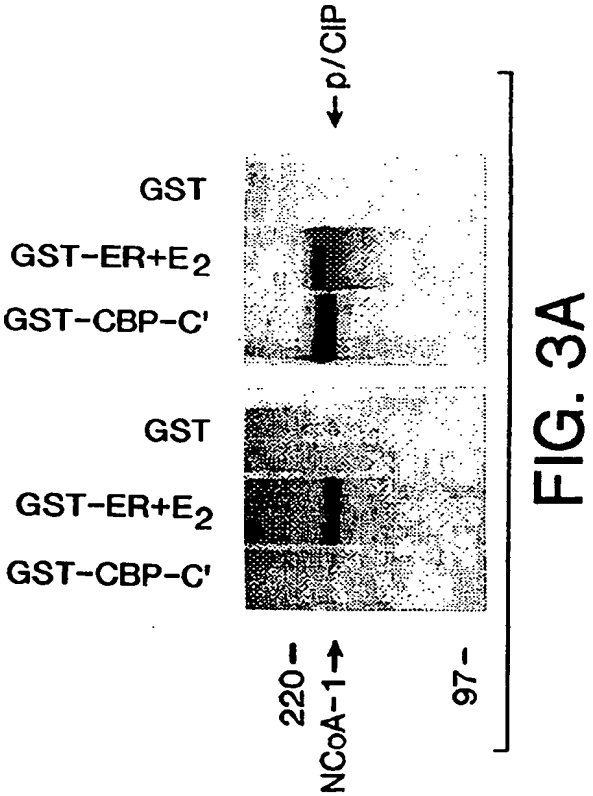

FIG. 3A

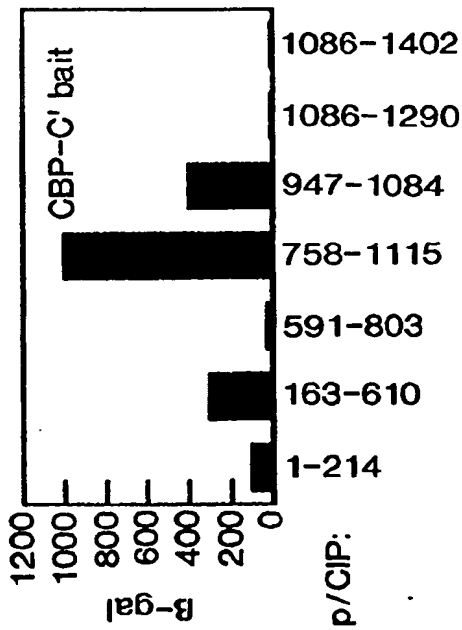

FIG. 3C-1

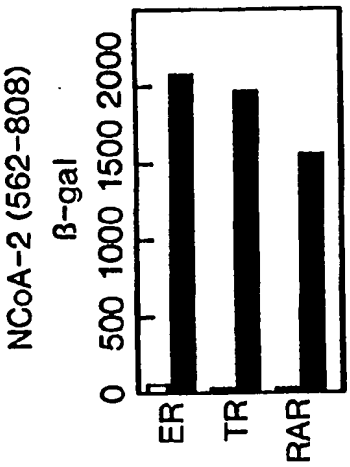

FIG. 3D-3

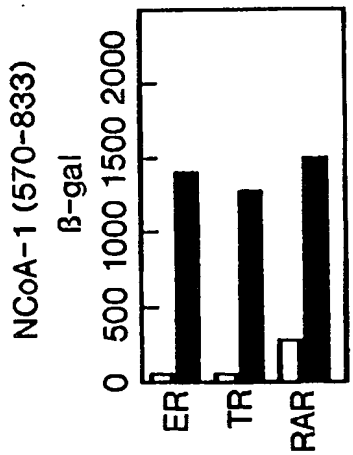

FIG. 3D-2

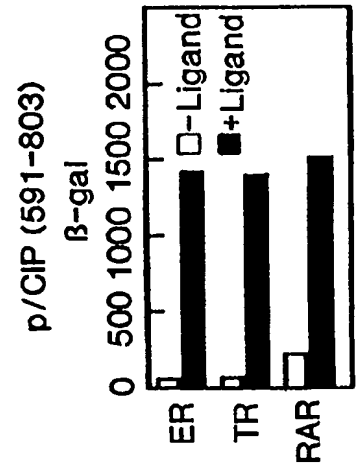

FIG. 3D-1

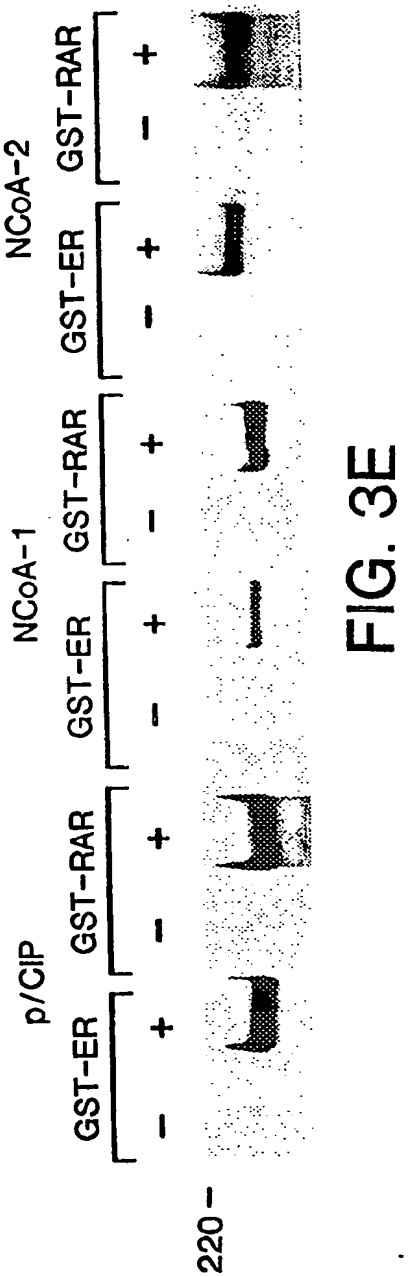

FIG. 3E

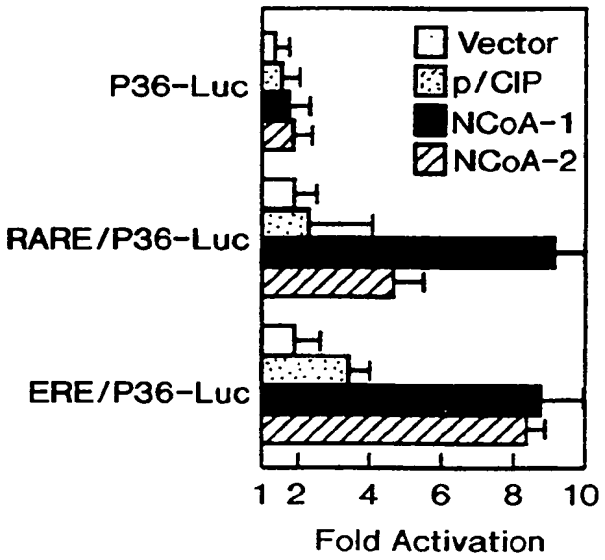

FIG. 3F-1

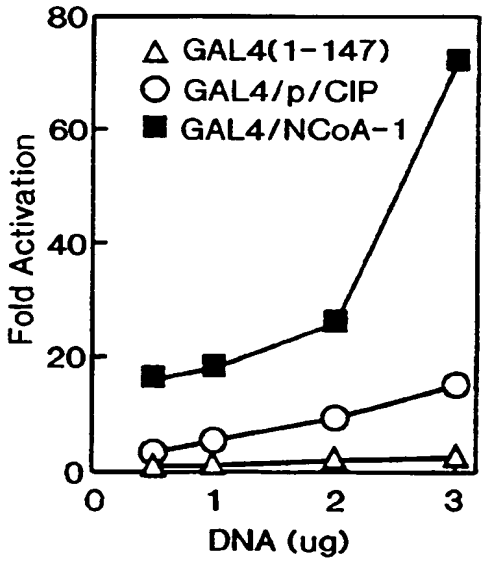

FIG. 3F-2

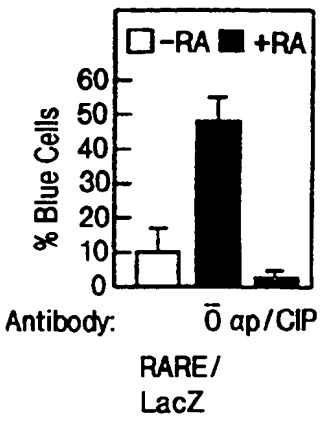

FIG. 4A-1

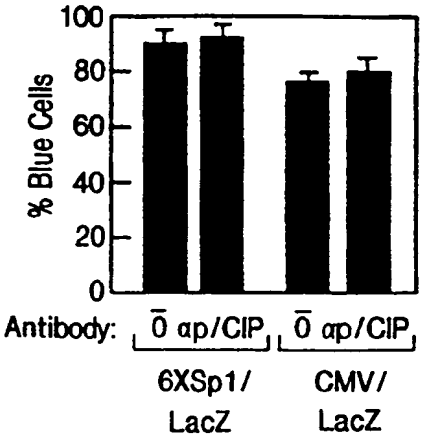

FIG. 4A-2

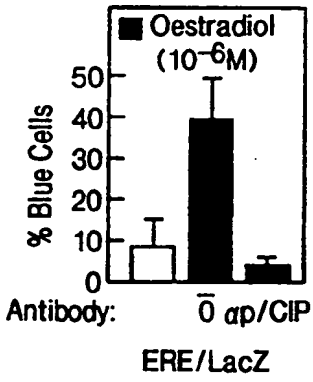

FIG. 4B-1

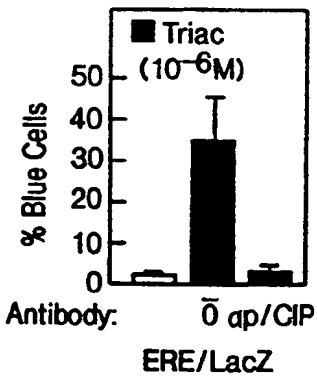

FIG. 4B-2

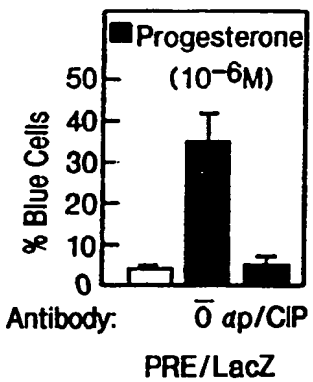

FIG. 4B-3

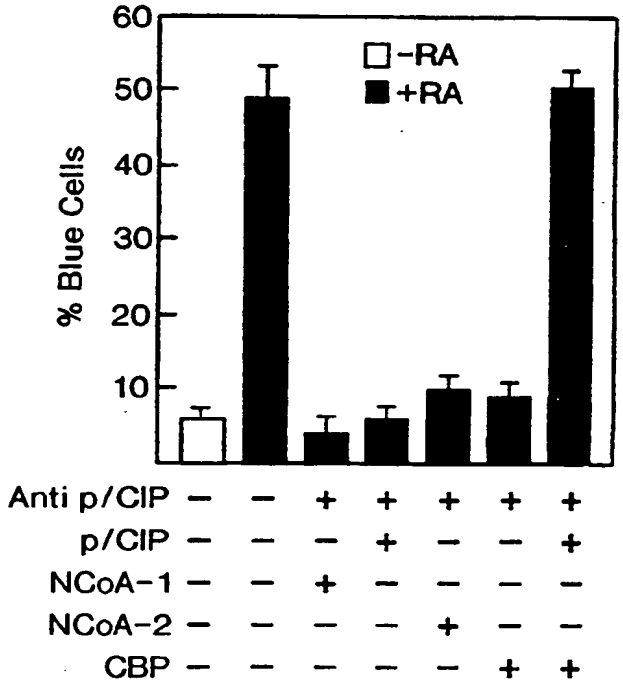

FIG. 4C

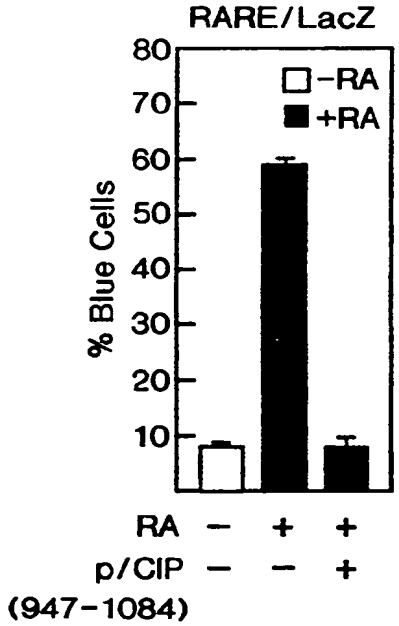

FIG. 4D-1

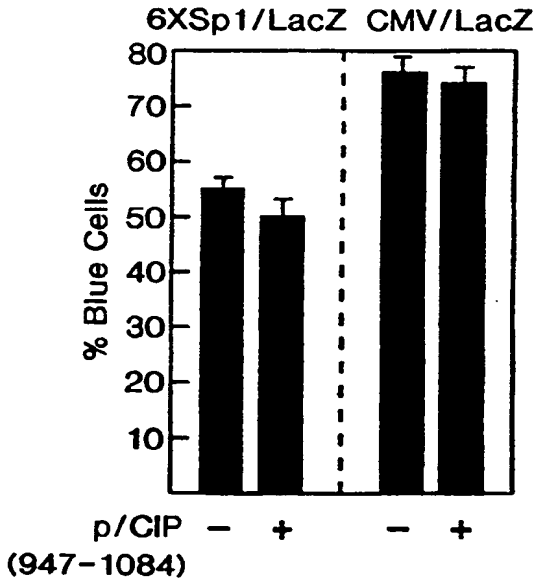

FIG. 4D-2

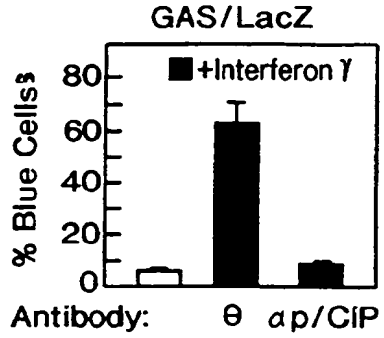

FIG. 4E-1

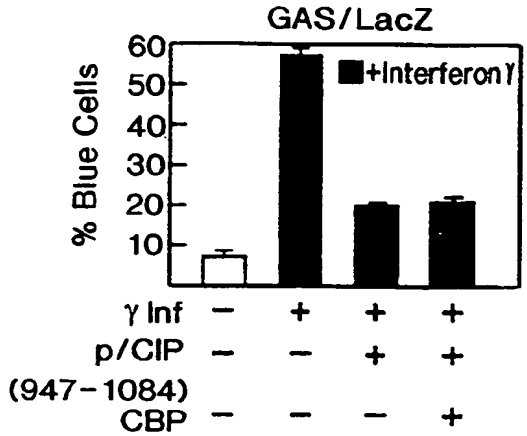

FIG. 4E-2

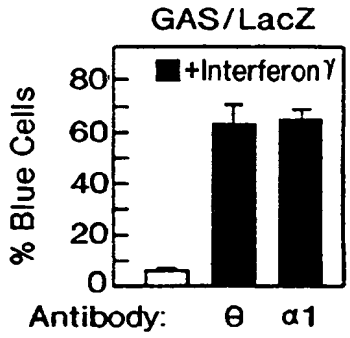

FIG. 4F-1

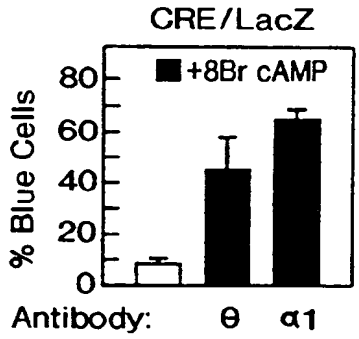

FIG. 4F-2

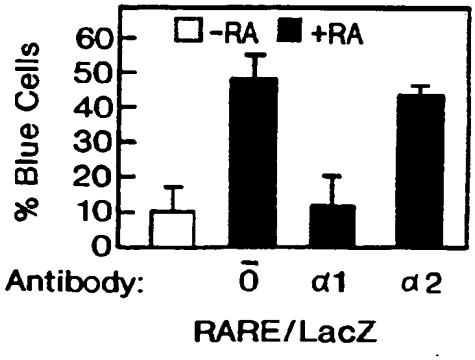

FIG. 5A-1

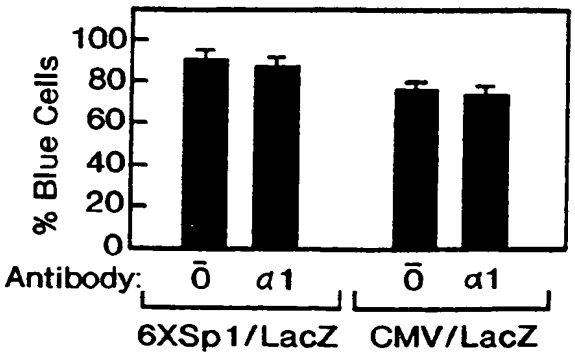

FIG. 5A-2

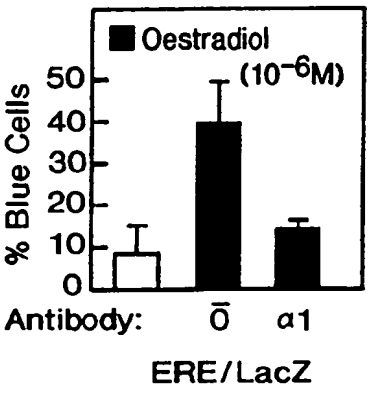

FIG. 5B-1

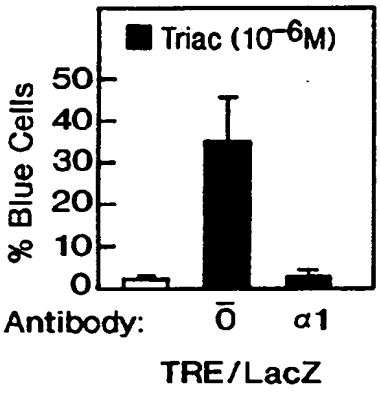

FIG. 5B-2

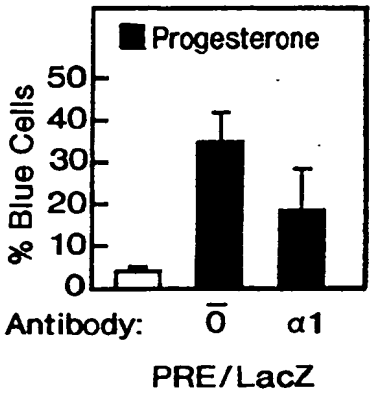

FIG. 5B-3

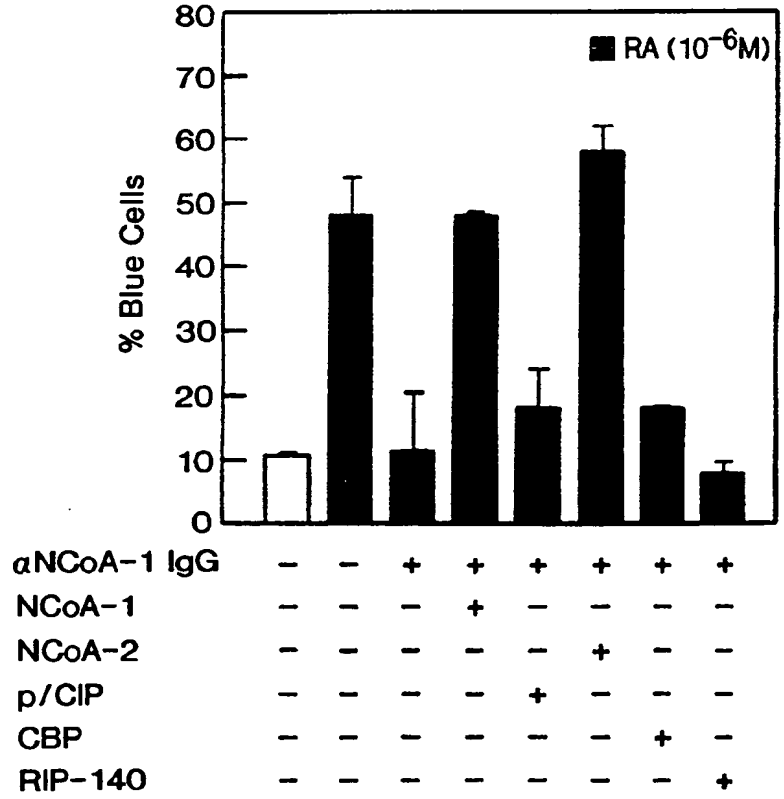

FIG. 5C

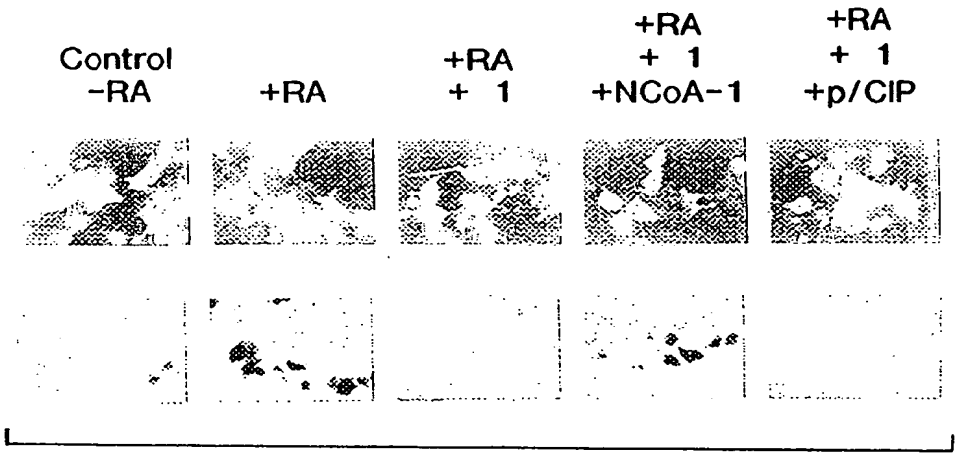

FIG. 5D

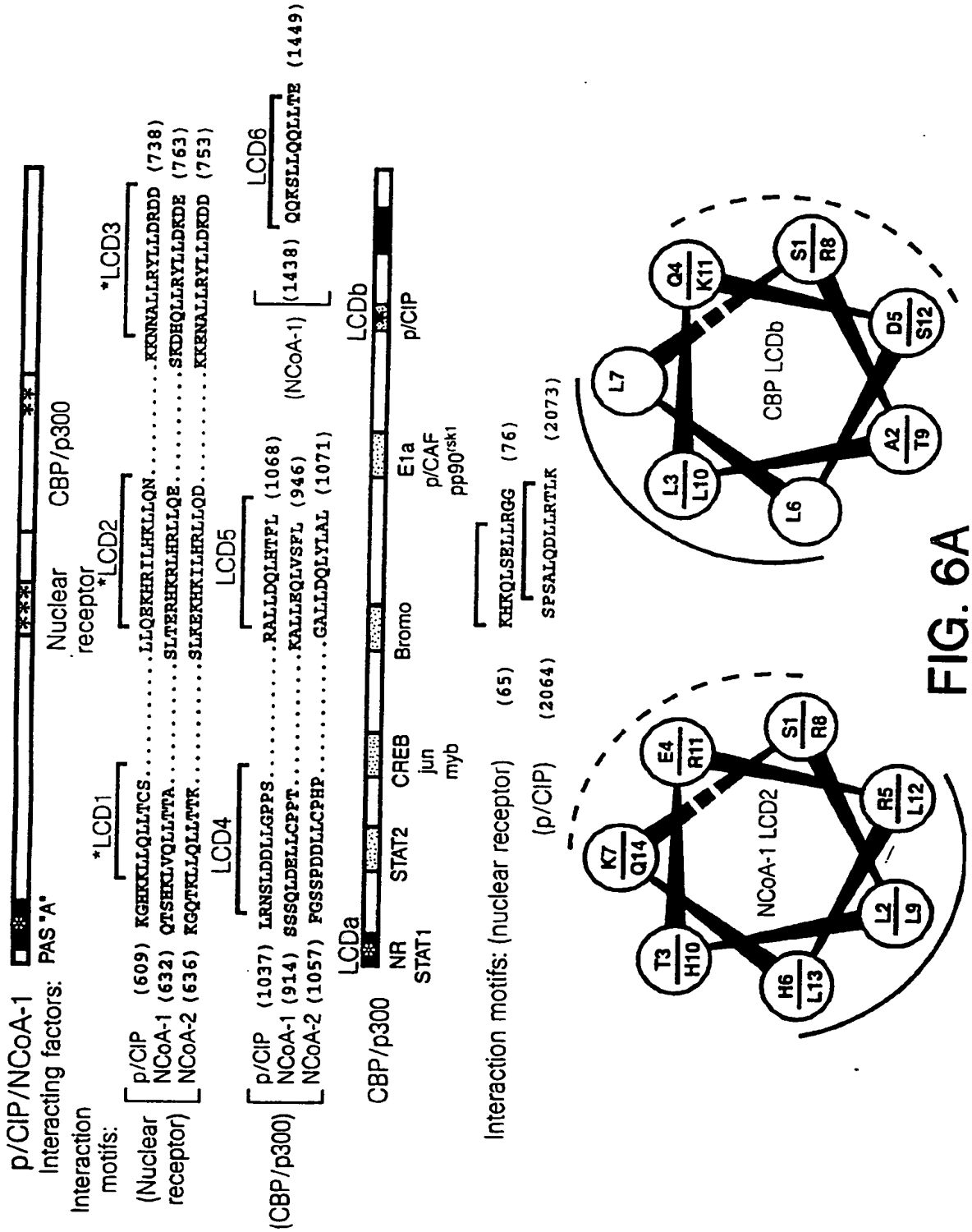
p/CIP/NCoA-1
Interacting factors:
PAS "A"
Nuclear receptor
CBP/p300
Interaction motifs:
(Nuclear receptor)
p/CIP (609) KGHKKLLQLLTCS
NCoA-1 (632) QTSHKLVQLLTTA
NCoA-2 (636) KGQTKLQLLTTK
*LCD1
*LCD2
*LCD3
LLQEKHRILHKLLQN (738)
SLTERHKRLHRLLQE (763)
SLKEKHKILHRLLQD (753)
KKNALLRYLLDRDD
SKDEQLLRYLLDKDE
KKENALLRYLLDKDD
LCD4
LCD5
LCD6
(CBP/p300)
p/CIP (1037) LRNSLDDLLGPPS
NCoA-1 (914) SSSQLDELLCPPT
NCoA-2 (1057) PGSSPDDLLCPHP
RALLDQLHTPL (1068)
KALLEQLVSFL (946)
GALLDQLYLAL (1071)
(NCoA-1) (1438) QQKSLLQQLLTE (1449)
LCDa
LCDb
CBP/p300
NR
STAT1
STAT2
CREB
jun
myb
Bromo
E1a
p/CAF
pp90rsk1
p/CIP
Interaction motifs: (nuclear receptor) (65) KHKQLSELLRGG (76)
(p/CIP) (2064) SPSALQDLLRTLK (2073)
NCoA-1 LCD2
CBP LCDb
K7/Q14
E4/R11
S1/R8
R5/L12
L2/L9
H6/L13
T3/H10
Q4/K11
S1/R8
D5/S12
A2/T9
L6
L3/L10
L7

FIG. 6A

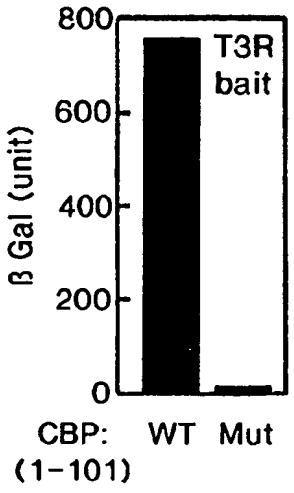

FIG. 6B

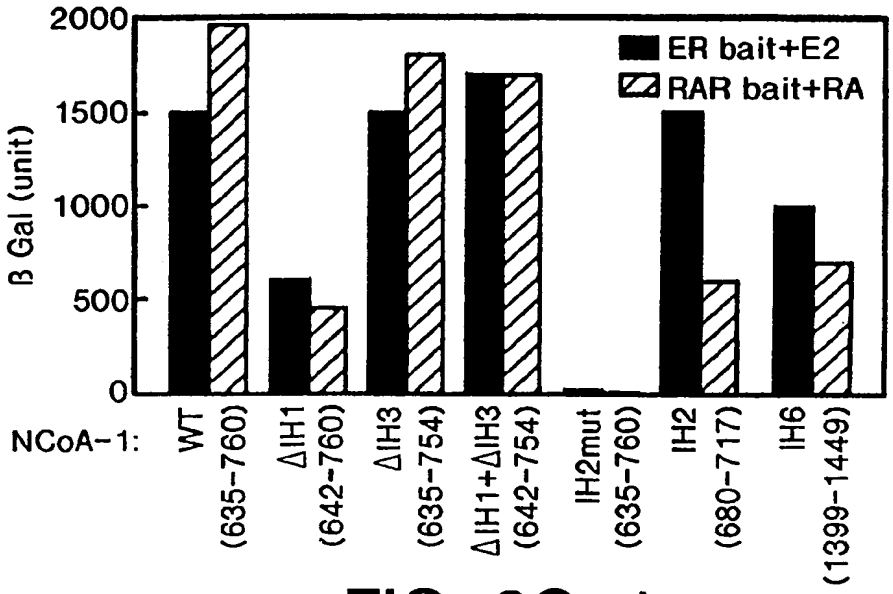

FIG. 6C-1

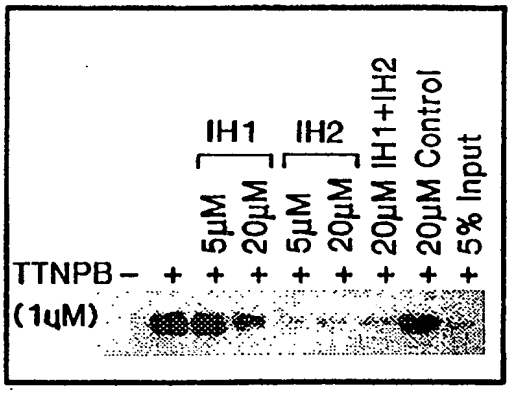

FIG. 6C-2

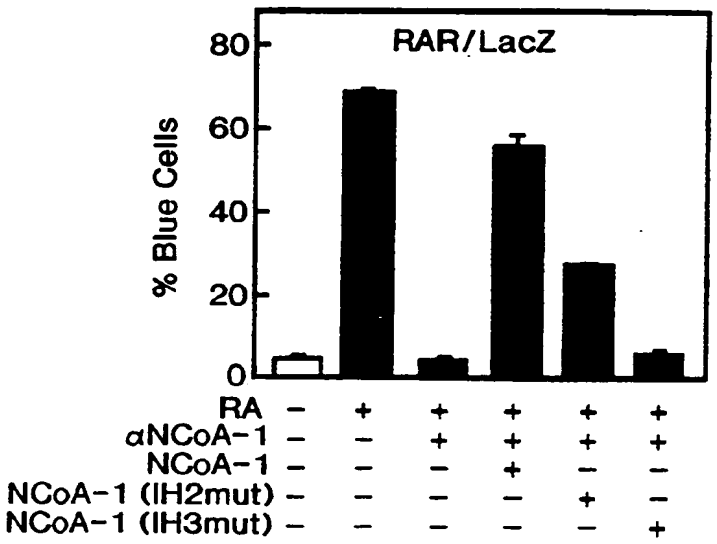
RAR/LacZ
% Blue Cells
80
60
40
20
0
RA – + + + + +
αNCoA-1 – – + + + +
NCoA-1 – – – + – –
NCoA-1 (IH2mut) – – – – + –
NCoA-1 (IH3mut) – – – – – +

FIG. 6D

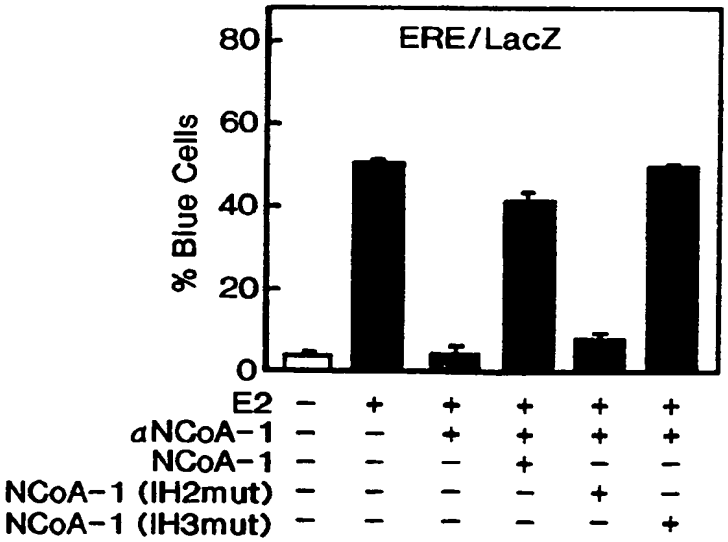
ERE/LacZ
% Blue Cells
80
60
40
20
0
E2 – + + + + +
αNCoA-1 – – + + + +
NCoA-1 – – – + – –
NCoA-1 (IH2mut) – – – – + –
NCoA-1 (IH3mut) – – – – – +

FIG. 6E

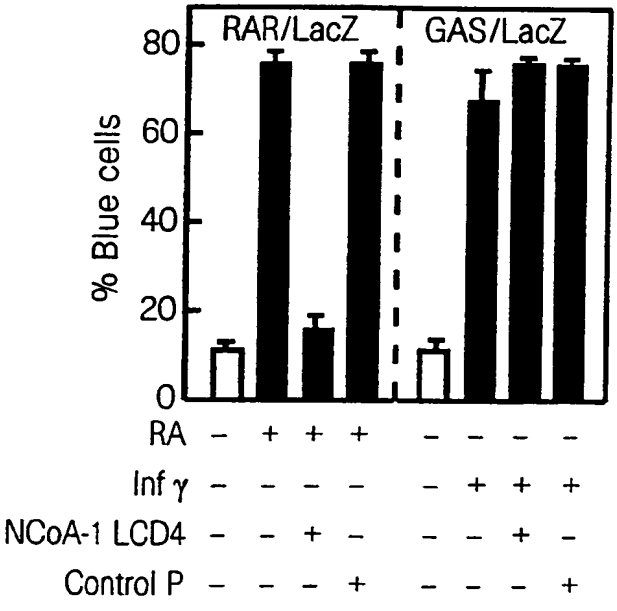

FIG. 7A

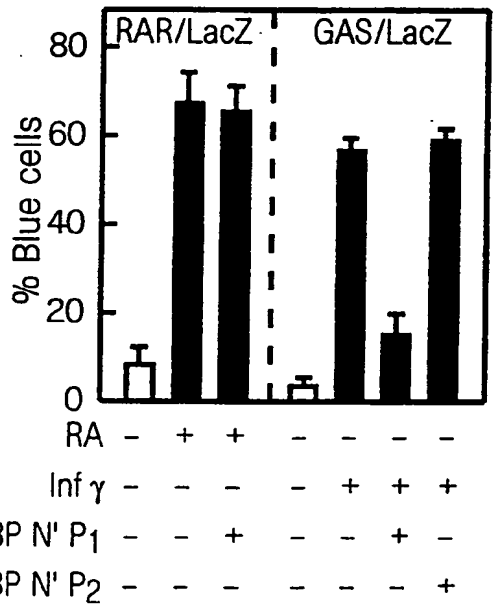

FIG. 7B

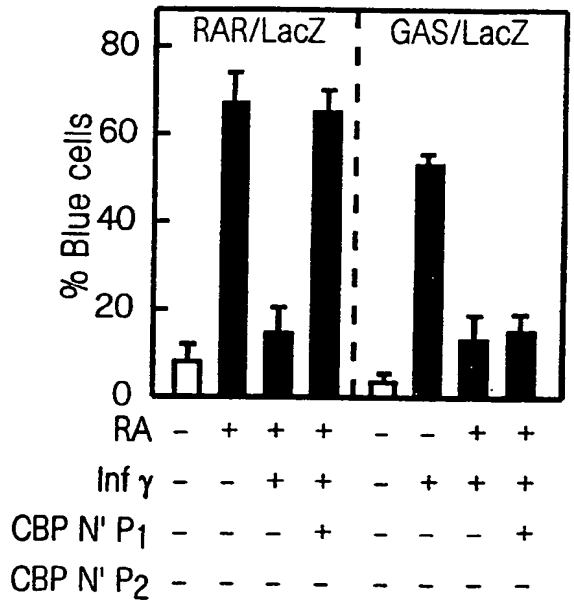

FIG. 7C

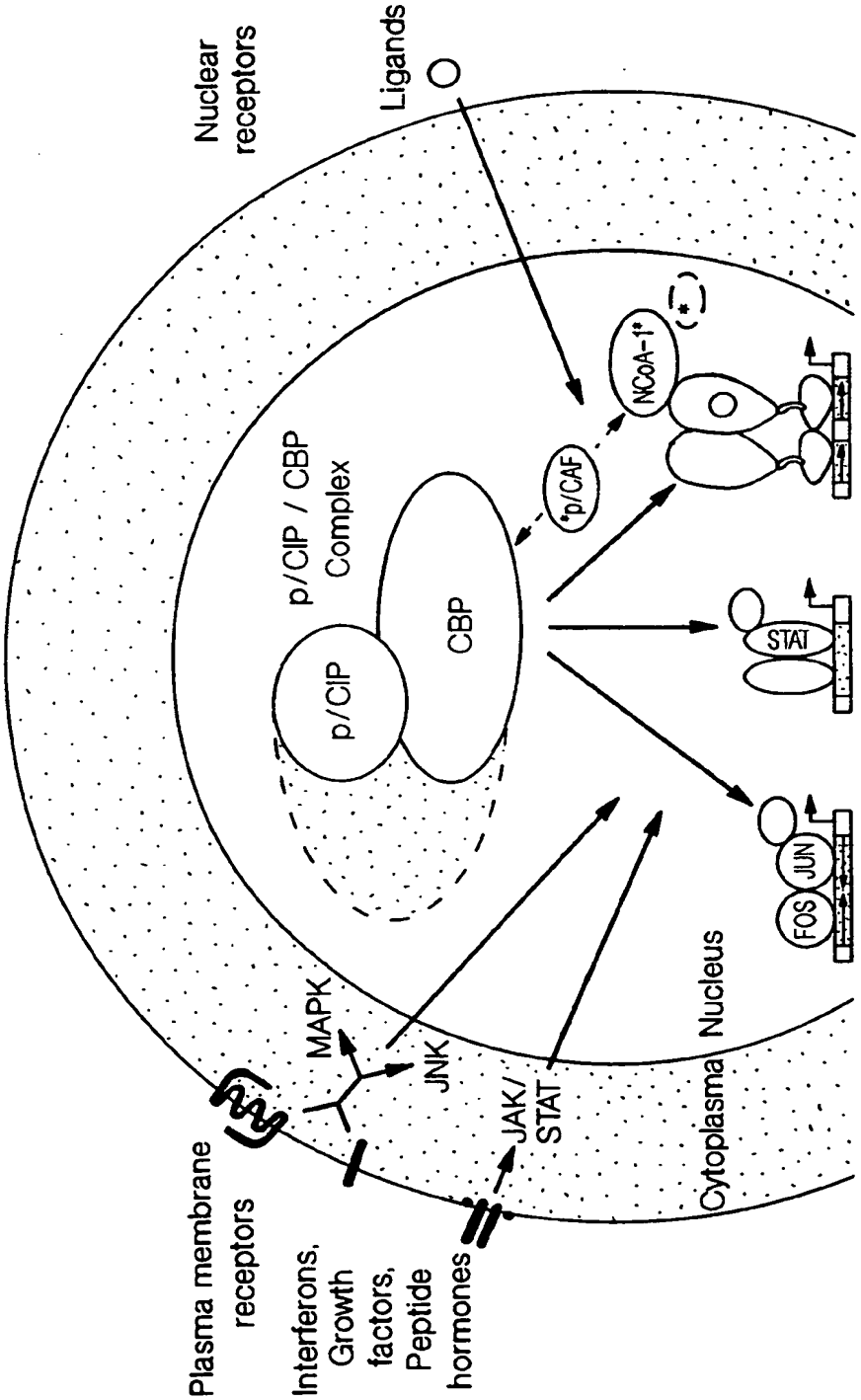
Nuclear receptors
Ligands
p/CIP / CBP Complex
p/CIP
CBP
p/CAF
NCoA-1
STAT
FOS
JUN
MAPK
JNK
JAK/ STAT
Nucleus
Cytoplasma
Plasma membrane receptors
Interferons, Growth factors, Peptide hormones

FIG. 7D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12263

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C07K 14/00; C12N 15/12; G01N 33/53
US CL :435/7.1; 530/350; 36/23.5, 24.31, 24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1; 530/350; 36/23.5, 24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Medline, Biosis, Embase CAPlus, WPIDS
Search terms: p/CIP, NCoA-2, coactivator

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P --- Y,P	TORCHIA et al. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature. 12 June 1997, Vol. 387, No. 6634, pages 677-684, see entire document.	1-25 --- 26-32
X,P --- Y,P	CHEN et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell. 08 August 1997, Vol. 90, pages 569-580, see entire document.	1,2,4,7-9,15-17,20,21 --- 5,6,18,19,26,28-31
X,P --- Y,P	LI et al. RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc. Natl. Acad. Sci. USA. August 1997, Vol. 94, pages 8479-8484, see entire document.	1,2,15,16 --- 4-9,17-21,26,28-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 AUGUST 1998	Date of mailing of the international search report 24 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT SCHWARTZMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12263

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HONG et al. GRIP1, a transcriptional coactivator for the AF2 transactivation domain of steroid, thyroid, retinoid, and Vitamin D receptors. Mol. Cell. Biol. May 1997, Vol. 17, No. 5, pages 2735-2744, see entire document.	1-32